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VOLUME 21, No. 5

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IN ENGLISH TRANSLATION

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ABBREVIATIONS MOST FREQUENTLY ENCOUNTERED IN RUSSIAN BIO-SCIENCES LITERATURE

Abbreviation (Transliterated)	Significance
AMN SSSR	Academy of Medical Sciences, USSR
AN SSSR	Academy of Sciences, USSR
BIN	Biological Institute, Botanical Institute
FTI	Institute of Physiotherapy
GONTI	State United Sci-Tech Press
GOST	All Union State Standard
GRRRI	State Roentgenology, Radiology, and Cancer Institute
GTTI	State Technical and Theoretical Literature Press
GU	State University
I Kh N	Scientific Research Institute of Surgical Neuropathology
IL (IIL)	Foreign Literature Press
IONKh	Inst. Gen. and Inorganic Chemistry (N. S. Kurnakov)
IP	Soil Science Inst. (Acad. Sci. USSR)
ISN (Izd. Sov. Nauk)	Soviet Science Press
Izd.	Press
LEM	Laboratory for experimental morphogenesis
LENDVI	Leningrad Inst. of Dermatology and Venereology
LEO	Laboratory of Experimental Zoology
LIKhT	Leningrad Surgical Institute for Tuberculosis and Bone and Joint Diseases
LIPZ	Leningrad Inst. for Study of Occupational Diseases
LIPK	Leningrad Blood Transfusion Institute
Medgiz	State Medical Literature Press
MOPISh	Moscow Society of Apiculture and Sericulture
MVI	Moscow Veterinary Institute
MZdrav	Ministry of Health
MZI	Moscow Zootechnical Institute
LOKhO	Leningrad Society of Orthopedic Surgeons
NIIZ	Scientific Research Institute of Zoology
NINKhI	Scientific Research Institute of Neurosurgery
NIU	Scientific Institute for Fertilizers
NIUIF	Scientific Research Institute of Fertilizers and Insecticides
NIVI	Veterinary Scientific Research Institute
ONTI	United Sci. Tech. Press
OTI	Division of Technical Information
RBO	Russian Botanical Society
ROP	Russian Society of Pathologists
SANIIRI	Central Asia Scientific Research Institute of Irrigation
SANIISh	Central Asia Scientific Research Institute of Sericulture
TsNII	All-Union Central Scientific Research Institute
TsNTL	Central Scientific and Technical Laboratory
VASKhNIL	All-Union Academy of Agricultural Sciences
VIG	All-Union Institute of Helminthology
VIEM	All-Union Institute of Experimental Medicine
VIR	All-Union Institute of Plant Cultivation
VIUAA	All-Union Institute of Fertilizers, Soil Science, and Agricultural Engineering
VIZR	All-Union Institute of Medical and Pharmaceutical Herbs
VNIRO	All-Union Scientific Institute of Fishing and Oceanography
ZIN	Zoological Inst. (Acad. Sci. USSR)

Note: Abbreviations not on this list and not explained in the translation have been transliterated, no further information about their significance being available to us. - Publisher.

PAPER-CHROMATOGRAPHIC STUDY OF THE STRUCTURE OF CARBOHYDRATES OF DIPHTHERIA BACTERIA

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During the past ten years the method of paper chromatography has obtained extensive use in the study of the composition and structure of polysaccharides of varying origin. The chief advangage of this method – the small amount of material required for analysis – makes it especially valuable in the study of polysaccharides of bacterial origin.

Concerned with work in this field are the studies of Barker and Bourne on the composition of the polysaccharide synthesized by Neisseria perflaga from sucrose [1], and on the oligosaccharide synthesized by Escherichia coli from maltose [2], carried out by use of the method of paper chromatography. Forsyth [3] investigated the composition of dextran synthesized by Leuconostoc mesenteroides from sucrose. By the same method Kent [4] investigated the polysaccharide composition of type II pneumococcus, Eagon [5] the structure of the capsular polysaccharide of Pseudomonas fluorescens and Evans [6] the capsular polysaccharide of Cryptococcus neoformans. Studies of Holdsworth [7] on the composition of the carbohydrate component of the cell walls of Corynebacterium diphtheriae, carried out by the method of paper chromatography, have confirmed the data we obtained previously [8] by use of classical analytical methods. In recently published work of Dziulynska and Mikulaszek [9], data are given on the chromatographic investigation of an entire series of bacterial polysaccharies: Shigella, Salmonella, Klebsiella, Pseudomonas, Streptococcus, Micrococcus, Bacillus erysipelotrix, Mycobacterium, Corynebacterium.

Together with obtaining an entire series of new data on the structure of carbohydrates, the extensive use of the method of paper chromatography in identification of hydrolysis products of methylated starch, glycogen and araban makes it necessary to reconsider a number of previously held views on the structure of these substances.

The purpose of the studies we have made is to check by the method of paper chromatography a number of views which we expressed previously [10] on the basis of study of the course of hydrolysis of various carbohydrate fractions of diphtheria bacteria.

Thus, studying the course of hydrolysis of mannan of diphtheria bacteria [10], we found that Barfoed's reaction becomes positive only upon hydrolysis for more than one hour, while reducing properties (Fehling's reaction) appear after 20 minute hydrolysis. On the basis of this we postulated that appearance of reducing capacity in the first hour of hydrolysis is related not to splitting off of free molecules of mannose, but to breakdown of mannan into molecules of oligosaccharides with reducing properties.

To check this view the following experiments were performed: 25 mg of a mannan preparation was dissolved in 2.5 ml 1N H_2SO_4 and the solution poured into ampouls, 0.2 ml in each. The ampouls were sealed and heated in a boiling water bath for various lengths of time: 1, 5, 15, 45 minutes, 1, 3, 5, 7 and 9 hours. Immediately after hydrolysis the contents of each ampoul were neutralized to litmus with dry barium carbonate and centrifuged directly in the ampouls. In order that chromatography of all the hydrolyzates obtained should proceed under identical conditions, all ten spots were placed on one sheet of paper (60 x 35 cm) with intervals of 5 cm between spots. Chromatographic paper of the Leningrad plant No. 2 was used. The nuetralized hydrolyzate was placed in the amount of 0.02 ml in a horizontal line at a distance of 5 cm from the lower edge of the paper in 2 portions

of 0.01 ml from a micropipet. The paper, rolled into a cylinder, was put into a vessel with convex inside bottom of internal diameter 24.5 cm and height 40 cm, into which was poured an upper layer of butanol-acetic mixture (acetic acid, butyl alcohol, water – 1 : 4 : 5). The solution flowed around the circumference of the cylinder walls in the form of a layer 5-6 cm in height; the amount used did not exceed 150 ml. The lower (aqueous) layer was put in the center of the vessel in a flat dish, so that it was inside the paper cylinder. The glass cylinder was closed with ground glass, and, when it was necessary to set up a taller chromatogram, with the inverted lower part of a desiccator of suitable size. Along the line of union of cylinder with cover was applied a strip of adhesive plaster. Two chromatograms were set up in parallel for development by different reagents. Chromatograms were kept in the vessel 36 hours, after which the sheets were removed and dried in air until the acetic acid odor disappeared. One chromatogram was developed with aniline hydrophthalate according to Partridge [11], the other with the silver reagent according to Trevelyan [12].

On the chromatogram developed with aniline hydrophthalate (Fig. 1) a series of spots is clearly seen, located at a level corresponding to mannose, in which the intensity of the spots increases with increase in time of hydrolysis.

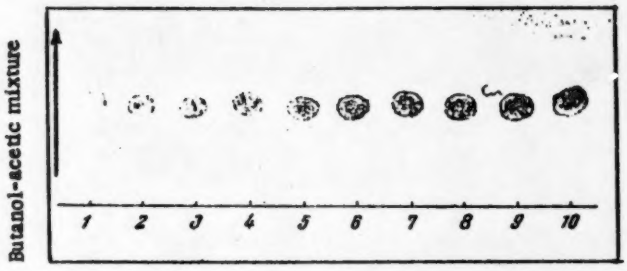

Fig. 1. Chromatogram of mannan hydrolyzates with development by aniline hydrophthalate: Hydrolysis time: 1) 1 min. 2) 5 min. 3) 15 min. 4) 30 min. 5) 45 min.; 6) 1 hr. 7) 3 hr. 8) 5 hr. 9) 7 hr. 10) 9 hr.

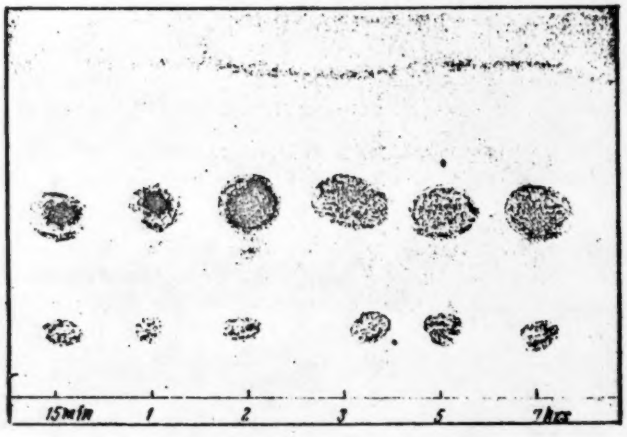

Fig. 2. Chromatogram of mannan hydrolyzates with development by silver reagent.

On the chromatogram developed with silver (Fig. 2) a series of weak spots, located at the level of disaccharides, corresponds to spots of mannose.

These data do not support the results obtained previously with respect to the time of appearance of molecules of free mannose, but do support the appearance of reducing oligosaccharides from the very beginning of hydrolysis.

The second carbohydrate component investigated was a polysaccharide, composed of arabinose, galactose and mannose, which we have called mannogalactoaraban. In studies carried out previously on the course of hydrolysis [13] the sequence of splitting off of sugars was determined by change in the amount of fermentable sugars in the hydrolyzate. It was found that arabinose splits off first in the course of hydrolysis (in 5 minutes), then galactose (in 15 minutes) and, finally, in 30 minutes – mannose.

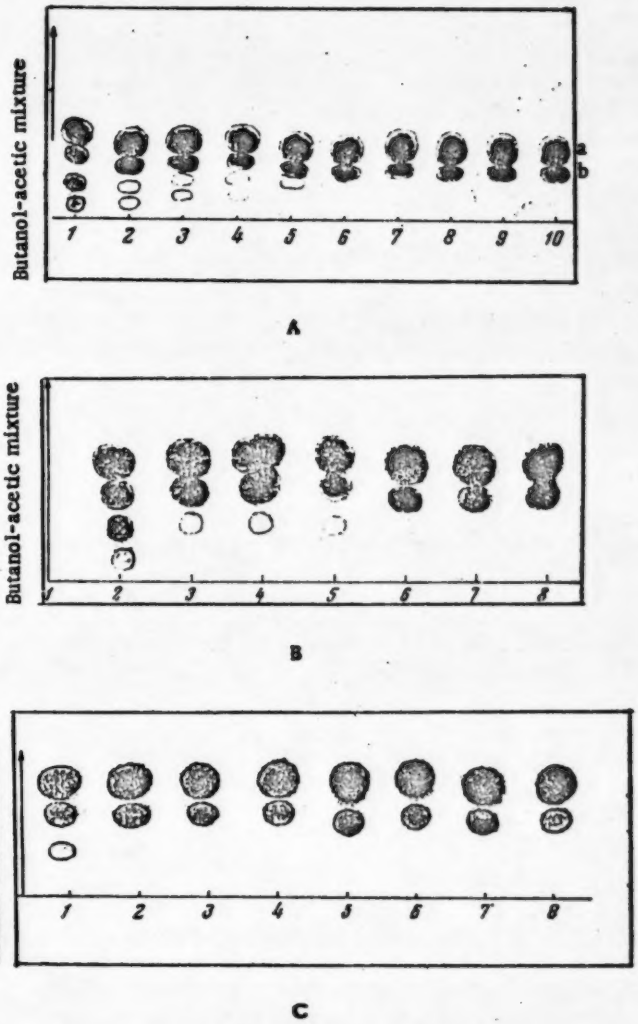

Fig. 3. Chromatograms of mannogalactoaraban hydrolyzates: A) with development by aniline hydrophthalate; a) arabinose; b) galactose; c) R_f = 0.085; e) R_f = 0.06; hydrolysis time: 1) 1 min.; 2) 5 min.; 3) 15 min.; 4) 30 min.; 5) 45 min.; 6) 1 hr.; 7) 3 hr.; 8) 4 hr.; 9) 5 hr.; 10) 6 hr.; B) with development by silver reagent; hydrolysis time: 1) 0; 2) 1 min.; 3) 5 min.; 4) 15 min.; 5) 30 min.; 6) 1 hr.; 7) 2 hr.; 8) 4 hr.; C) unneutralized hydrolyzate, developed with aniline hydrophthalate; hydrolysis time: 1) 1 min.; 2) 5 min.; 3) 15 min.; 4) 30 min.; 5) 1 hr.; 6) 2 hr.; 7) 3 hr.; 8) 4 hr.

To check these data we also used the method of ascending paper chromatography. The combination of components in this polysaccharide is unsuitable for chromatographic separation, since mannose and arabinose have the same R_f value in butanol-acetic mixture, while in phenol galactose and mannose have the same R_f value.

An attempt to use, as the mobile phase, a mixture consisting of ethyl acetate, acetic acid and water in the proportions 3:1:3 did not give the expected results. In order to obtain complete separation of the three components, we varied the experimental conditions. However, with development of such chromatograms by aniline hydrophthalate we obtained only two spots, regardless of experimental conditions.

Use of butanol-acetic mixture as the mobile phase with subsequent development by aniline hydrophthalate nevertheless answered the question concerning the time of splitting off of arabinose and galactose, since in this solvent they have a different R_f value and are developed in the form of spots of different color (arabinose – red spot, galactose – brown).

For the three-component polysaccharide we increased the concentration of the initial solution so that the mannose concentration was 1%, since the amount of this sugar in the polysaccharide is the least, and according to our data [9], is ~ 20%. With this purpose, 130 mg of mannogalactoaraban preparation was dissolved in 2.6 ml of 1 N H_2SO_4. Hydrolysis and neutralization were performed just as for mannan. Time of hydrolysis: 1, 5, 15, 30, 45 minutes, 1, 3, 4, 5, 6 hours. The neutralized hydrolyzates were placed on a sheet of paper in the amount of 0.01 ml for each spot and were kept in butanol-acetic mixture for 36 hours. Two chromatograms were set up in parallel, one of which was developed with aniline hydrophthalate, the other with silver reagent.

On the chromatogram developed with aniline hydrophthalate two rows of spots are clearly visible – an upper row of spots of cherry-red color – arabinose (R_f = 0.21) and a lower row of spots of brown color – galactose (R_f = 0.15) (Fig. 3A). The same result was also obtained on the chromatogram developed with silver (Fig. 3,B). On this chromatogram were placed: the initial polysaccharide solution in 1N H_2SO_4 (point 1) and hydrolyzates after 1, 5, 15, 30 minutes, 1, 2, and 4 hours of hydrolysis.

A weakly colored spot characteristic of the level of disaccharides (R_f = 0.085), visible on both chromatograms but more distinctly on the chromatogram developed with silver, is also observed for solutions hydrolyzed less than one hour. Together with this spot there is also observed a fourth spot with R_f = 0.06.

Since the process of neutralization of the hydrolyzate, even when carried out directly in the ampouls, is associated with a loss of the solution, which is of importance in cases of insufficiency of material, we tried to eliminate it.

With this purpose 20 mg of the preparation was dissolved in 0.4 ml 1N HCl, the solution poured into ampouls, 0.05 ml in each, and hydrolyzed 1, 5, 15, 30 minutes, 1, 2, 3 and 4 hours. The hydrolyzates without preliminary neutralization were placed on two sheets of paper 45 x 53 cm and kept 32 hours in butanol-acetic misture. Of two parallel chromatograms one was developed with aniline hydrophthalate, in which very good results were obtained (Fig. 3, B).

With development by silver the results were not as good.

The chromatographic data (Fig. 3) indicate that from the very beginning of hydrolysis (1 minute) arabinose and galactose are observed in the solution.

To answer the question concerning the time of splitting off of mannose we had to resort to two-dimensional chromatography. The concentration of polysaccharide was increased: 75 mg in 1.5 ml 1 N H_2SO_4. The solution was poured into ampouls, 0.2 ml in each, and hydrolyzed 5, 15, 30 minutes, 1 hour and 2 hours, then neutralized and centrifuged in the ampouls. Each of the neutralized hydrolyzates was placed in the amount of 0.2 ml in the left corner of a sheet of paper 45 x 50 cm at a distance of 4 cm from the lower and left edges of the paper. The sheet, rolled into a cylinder, was put for 32 hours into phenol, saturated with water, after which it was kept at room temperature until the phenol odor completely disappeared. Then the sheet was rotated 90° and placed for the same length of time in butanol-acetic mixture. After being dried, one of the two parallel chromatograms was developed with aniline hydrophthalate, the other with silver. In this case the advantage of development with aniline hydrophthalate is shown particularly clearly. With such development there are distinctly visible, on chromatograms of hydrolyzates obtained with a hydrolysis time greater than 15 minutes, three spots characteristic of arabinose, galactose and mannose. But on chromatograms developed with silver the arabinose and mannose spots frequently are run together into one large extended spot.

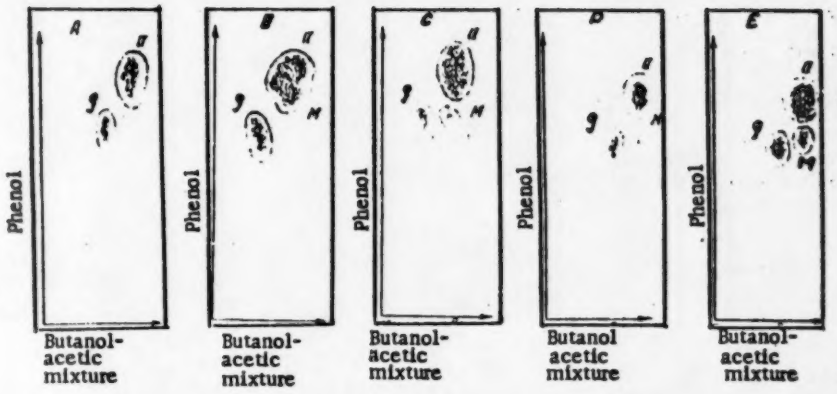

Fig. 4. Two-dimensional chromatograms of mannogalactoaraban hydrolyzates with development by aniline hydrophthalate: Duration of hydrolysis: A) 5 min.; B) 15 min.; C) 30 min.; D) 1 hr.; E) 2 hr. a) Arabinose; g) galactose; m) mannose.

In Fig. 4 are shown photographs of five chromatograms developed with aniline hydrophthalate. The results obtained indicate that arabinose and galactose are split off simultaneously in the first minutes of hydrolysis, while mannose is split off only upon continuation of hydrolysis for approximately 15 minutes. Evidently owing to the significant predominance of arabinose over galactose in the fermentation method these monosaccharides did not appear simultaneously.

SUMMARY

The paper chromatography method permits detection of free monosaccharides in hydrolyzates of complex carbohydrates much earlier than do the generally used qualitative reactions. Development with aniline hydrophthalate has an especially great advantage in cases where one of the structural components of the polysaccharide is a pentose.

In hydrolysis of mannan of diphtheria bacteria, from the first minutes of hydrolysis are split off free mannose (R_f = 0.22) and an oligosaccharide located at a level corresponding to disaccharides (R_f = 0.10).

In hydrolysis of mannogalactoaraban of the same origin, after the first minutes of hydrolysis are split off arabinose (R_f = 0.21) and galactose (R_f = 0.15), while mannose appears after 15-minute hydrolysis. Together with monosaccharides, after hydrolysis of less than one hour are found oligosaccharides with value of R_f = 0.085 (level of disaccharides) and after hydrolysis of less than 15 minutes – oligosaccharides with value of R_f = 0.06.

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MICROELECTROPHORETIC STUDY OF THE PROTEIN COMPOSITION OF MUSCLE TISSUE IN ONTOGENESIS

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Study of the protein composition of organs and tissues in ontogenesis permits determination of the biochemical changes at the different stages of development which occur parallel to emergence and development of individual functions of the organism. From this point of view study of the protein composition of muscle acquires importance.

There are a number of studies devoted to investigation of the structure of embryonic muscle tissue and its content of water, nitrogen fractions, enzyme systems, etc. [1-3]. In the study of embryonic muscle tissue much attention has been directed to the contractile protein – actomyosin and its components: actin and myosin [4-8].

In the studies of one of the present authors [5] it was established that appearance and accumulation of actomyosin in human muscle, and also in muscle of embryos and newborn animals, occur at varying periods of embryonic and postembryonic development. A difference was found in the time of appearance of the actomyosin system between two groups of animals: first group – guinea pigs and hens, second group – mice, rats and rooks. The first group was characterized by a sudden appearance (rapid change) of the reactions of muscle proteins with adenosinetriphosphoric acid while still in the period of embryonic development ; the second group – by a relatively uniform increase in intensity of these reactions, which occurred only on the 10th-13th day of postembryonic development.

These differences in time of accumulation of contractile proteins are in conformity with the general biological features of both groups of animals. Thus, animals of the first group are born with vision, are capable of independent existence, are "born mature" [9]. Animals of the second group are born blind, are undeveloped and incapable of independent existence, are "born immature".

Considerably less studied are the changes of soluble proteins of muscle plasma (sarcoplasm) in the process of formation of the muscle fiber. Data in the literature [6, 7, 10, 11] on this question have a general character and do not permit a clear conception of the type of changes of the different proteins and enzyme systems of sarcoplasm in the process of ontogenetic development of the animal. However, for investigation of the processes of muscle tissue formation in ontogenesis, and the biochemical changes occurring at this time, the study of the soluble proteins of sarcoplasm, which play a large part in muscle metabolism, is undoubtedly of importance.

In this paper are reported the results of a microelectrophoretic study of the soluble proteins (aldolase, glyceraldehyde dehydrogenase, phosphorylase and myoalbumin) of the sarcoplasm of certain animals and birds. Studies were made on rabbits, rats, guinea pigs and hens at different periods of embryonic and postembryonic development.

According to data in the literature [12, 13], upon separation of the soluble proteins of sarcoplasm in the Tiselius apparatus there have been found three main fractions: I – (n + m + l), II – (K_1 + K_2) and III – (h + l), which on the basis of electrophoretic mobility were identified with specific muscle proteins. These data were subsequently reproduced with use of paper electrophoresis [14]. The distribution on the electrophoregram of

proteins with enzymic activity was also determined [15]. According to these data, m and l fractions show aldolase and triosephosphate dehydrogenase activity, and k fractions – phosphorylase activity.

METHODS

Muscle tissue (rabbit, rat, guinea pig, hen), carefully freed of fatty layers and fascia, was washed free of blood, cut up with scissors, and then ground in a glass homogenizer for 3 minutes. The homogenate was extracted with 1.5 volumes of phosphate buffer (pH 7.7; ionic strength – 0.1) for 2 hours with periodic stirring, then centrifuged at 5000-6000 revolutions per minute for 30 minutes. It was determined in preliminary experiments that dialysis of the extract does not influence the results of electrophoresis. The extract was kept at a temperature of ~ 2° not more than 7 days. All procedures associated with preparation of the extract except centrifugation were carried out in the cold (from 0 to 2°). It was shown that muscle tissue which has been frozen for a week can be used in the investigation.

Electrophoresis was carried out in a cell described previously [16, 17] at room temperature for 18-19 hours, at potential gradient 5-5.5 v/cm with phosphate buffer (pH 6.4; ionic strength 0.1). The extract was applied in the amount of 0.03 ml to the center of a vertically suspended strip of filter paper 40 x 4 cm. Quantitative determination was carried out by means of elution (0.1 N NaOH for 1 hour) of the portions of the electrophoregram corresponding to separate fractions of muscle proteins, with subsequent determination of color intensity of the eluates in a photoelectric colorimeter.

The data we obtained on the content of protein fractions in muscle extracts of rabbits, rats, guinea pigs and hens at certain periods of embryonic and postembryonic development are presented in Table 1 (average data from 4-6 determinations are given; deviations in individual determinations did not exceed 5%).

In analysis of these data for all animals investigated the attention is drawn to the increased content of phosphorylase (or protein with identical electrophoretic mobility) in embryonic muscle tissue in comparison with tissue of the adult animal. The latter circumstance is in conformity with the known specific features of the metabolism of this tissue. In embryonic muscle tissue of all animals investigated, in comparison with muscle of the adult animals there is a significantly higher content of myoalbumin (fraction h) – the most electrophoretically mobile fraction. In the process of the animal's development in proportion to maturity of the muscle the percentage content of myoalbumin sharply decreases (from 3 to 8 times), which is, apparently, a reflection of the consolidation of muscle protein molecules occurring in the process of ontogenetic development.

There are data which indicate the important role of myoalbumin in the formation of other proteins of the muscle fiber in the period of embryonic development [6]. Apparently the physiological significance of myoalbumin lies in the fact that it represents a reserve protein of muscle tissue, which is used for synthesis of structural proteins. This is indicated by the evident correlation between the process of accumulation of actomyosin, myosin and actin in muscle tissue of embryos and newborn animals [5] and the process of change of soluble protenis of sarcoplasm (decrease of myoalbumin fraction). In the period of sharp decrease of myoalbumin content in the muscle fiber there is observed an accumulation of actomyosin. This provides a basis for suggesting the possibility of conversion of globular to fibrillar proteins in the maturation process of muscle tissue.

It should be noted that fractions n, m amd l, containing a number of enzymes of glycogenolysis, are almost unseparated in electrophoresis of embryonic muscle tissue. This indicates that these enzymes not only are contained in embryonic muscle tissue in smaller amount, but, apparently, also have certain qualitative differences in comparison with these same muscle proteins of adult animals. Furthermore, there is noted a species difference in the content of certain proteins of sarcoplasm. Thus, for instance, the content of fractions n, m and l on electrophoregrams of muscle extracts of the adult rat and hen according to our data, averages 2.8, 16.5 and 52.1% respectively. However, on electrophoregrams of muscle extracts of the adult rabbit fractions n, m and l differ from each other in content considerably less; the content of each fraction varies within the limits 20-25% of the total amount of protein contained in the extract.

Of considerable interest are the data indicating differences in time of accumulation of the proteins investigated in sarcoplasm of rabbits and rats in comparison with guinea pigs and hens.

As is evident from Table 1, in sarcoplasm of the guinea pig by the end of the embryonic period of development there is observable a sudden accumulation (rapid change) of enzymes of glycolysis (aldolase and glycer-

aldehyde dehydrogenase). Thus, for instance, by the 53rd day of embryonic development this fraction represents 35.2%, and by the 64th day – 55.7% of the total amount of protein in the extract; the myoalbumin fraction is correspondingly lowered from 37.4 to 11.9%. The same relationship can be noted in study of the data obtained on chick embryos. Thus, while in the 15-day chick embryo the fraction n + m + 1 represents 20.6%, phosphorylase – 64.3%, and myoalbumin – 15.1%, in the 20-day embryo it is – 45.8, 42.4, and 11.8%, respectively. In other words, in the course of 4-5 days of embryonic development there is observable a sudden change in content of the fractions investigated. Let us recall that at this period in chick embryo muscle there has been noted an intense accumulation of proteins of the actomyosin complex. The relationship of the protein fractions in muscle extracts of the newborn guinea pig, and also of one-day-old chicks, approaches in type the relationship characteristic of muscle of the adult animals.

TABLE 1

Content of Protein Fractions in Muscle Extracts.
Ionic strength of extract, 0.1; average values in percent.

Animal species	Age of animal in days*	Aldolase + glyceraldehyde dehydrogenase n + m + 1	Phosphorylase k	Myoalbumin h
Rabbits	24 of embryonic development	25.2	43.7	31.1
	1 of postembryonic development	36.3	41.9	21.8
	14 of postembryonic development	62.5	29.5	8.0
	20 of postembryonic development	70.0	25.1	4.9
	Adult rabbit	71.8	23.2	5.0
Rats	20 of embryonic development	28.3	37.9	33.8
	1-2 of postembryonic development	32.3	38.9	28.8
	13 of postembryonic development	47.3	37.7	15.0
	18 of postembryonic development	57.5	31.9	10.6
	Adult rat	71.2	24.8	4.0
Guinea pigs	53 of embryonic development	35.2	27.4	37.4
	64 of embryonic development	55.7	32.4	11.9
	1-2 of postembryonic development	57.6	30.4	12.0
	14 of postembryonic development	68.5	20.3	11.2
	Adult guinea pig	72.7	22.4	4.9
Hens	15 of embryonic development	20.6	64.3	15.1
	20 of embryonic development	45.8	42.4	11.8
	1 of postembryonic development	53.2	36.0	10.8
	12 of postembryonic development	60.2	33.1	6.7
	Adult hen	69.6	25.0	5.4

*Age of animals was determined according to the tables of Needham [20]

In muscle extracts of rats there is noted another relationship in the intensity of change of the fractions studied. In these animals, in contrast to guinea pigs, in the first days of postembryonic development the relationship of the protein fractions still recalls the picture in embryonic tissue. Only during the first month of postembryonic life in rats does there occur a very slow increase of the aldolase and glyceraldehyde dehydrogenase (n + m + 1) fraction and a corresponding decrease of the remaining fractions (phosphorylase and myoalbumin). In comparing Fig. 1 and 2 one is struck by the sharp difference between electrophoregrams of muscle extracts of newborn rats and newborn guinea pigs. Whereas the muscle extract curve of newborn rats differs sharply from that of adult rats, the muscle extract curve of the newborn guinea pig differs insignificantly from the muscle

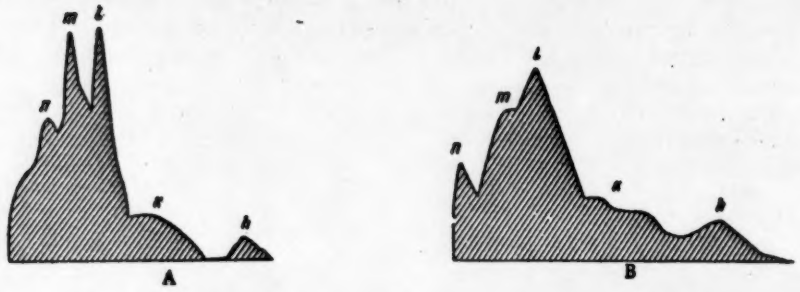

Fig. 1. Electrophoregram of muscle extract proteins of adult guinea pig (A) and of newborn guinea pig (B).

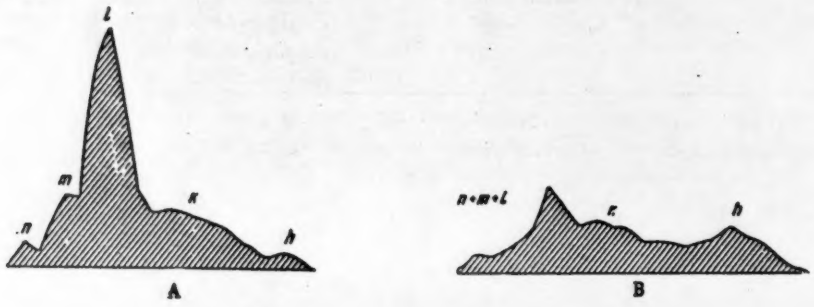

Fig. 2. Electrophoregram of muscle extract proteins of adult rat (A) and of newborn rat (B).

extract curve of the adult animal. The content of protein fractions in muscle extracts of newborn rabbits recalls the picture characteristic for rats (Fig. 3 and Table 1). Maturity of the enzyme systems of sarcoplasm is reached in rabbits, according to the electrophoresis data, essentially by the 20th day of postembryonic development. This is in agreement with data [18] in the literature that by the 10th-20th day of postembryonic development of rabbits the muscle content of ammonia, glutamine, glutamic acid, and also the activity of glutaminase, are decreased to the level characteristic of muscle tissue of the adult animals.

TABLE 2

Total Nitrogen Content in Muscle Extracts.
Extract with low ionic strength; average data in mg/ml

Animal species	In newborn animals	In adult animals
Rabbits	2.10	6.42
Rats	1.37	4.00
Guinea pigs	3.80	4.51
Hens	3.18	3.38

Together with study of muscle extracts by the paper electrophoresis method, their total nitrogen content was determined. This likewise provides a certain conception of the amount of sarcoplasm proteins in newborn animals in comparison with adults.

From Table 2 it is evident that the total nitrogen content in muscle extracts of newborn guinea pigs and one-day-old chicks differs little from the nitrogen content in muscle extracts of the adult guinea pig and hen, whereas the total nitrogen content in muscle extracts of newborn rats and rabbits is several times lower than in muscle extracts of the adult animals.

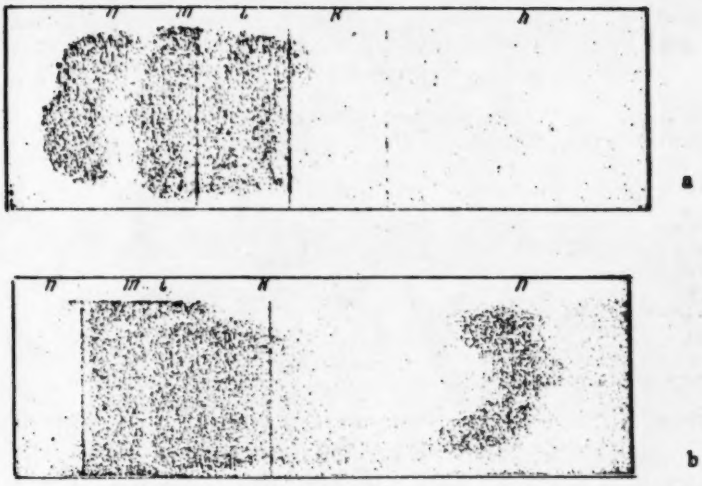

Fig. 3. Electrophoregram of muscle extract proteins of adult rabbits (a) and of newborn rabbits (b).

Our results permit the assumption that between animals "born mature" (guinea pigs and chicks) and those "born immature" (rats, rabbits) there exists considerable divergence in time of maturity and intensity of accumulation of the soluble proteins of sarcoplasm, which, apparently, reflects a difference in time of functional maturity of these groups of animals. Characteristic of the first group of animals ("born mature") is a considerable accumulation of aldolase and glyceraldehyde dehydrogenase and a correspondingly sharp decrease of the myoalbumin fraction in sarcoplasm at the end of the embryonic period of development. In animals of the second group ("born immature") these changes gradually occur only in postembryonic life.

This fact, as well as the differences we found earlier [5] in intensity of accumulation of actomyosin in various animals, indicates the presence of a certain parallelism between the maturity of a number of enzyme systems in muscle and the formation of certain physiological functions of the animal in the process of ontogenetic development. All this once again supports the correctness of the concept of the inseparability of the enzyme and function, of the fact that enzymes are an integral constituent part of functional physiological structures [19].

It should be noted that the method of paper electrophoresis of muscle proteins, requiring minimal amounts of tissue for study, presents wide possibilities for study of sarcoplasm proteins in the normal and in various pathological conditions.

Investigation of changes of the protein composition of muscle tissue in the process of ontogenesis permits us to study more profoundly the biochemical changes in muscle pathology, in particular in regeneration of muscle tissue after trauma in various age groups, and also to develop methods to influence effectively the process of regeneration.

We express thanks to M. V. Umanskaya for assistance in the study.

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PATHWAYS OF CONVERSION TO UREA OF NITROGEN OF VARIOUS AMINO ACIDS AND OF AMMONIA IN MAMMALIAN LIVER

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We [1] have recently shown that in rat liver homogenate during urea formation from citrulline (CTR) (that is, in the second stage of the ornithine cycle) various L- and D-amino acids and salts of ammonia can serve as sources of the second nitrogen atom of urea (conventionally: "N-donors").

In agreement with studies of Ratner [2, 3], only L-aspartic acid (AS) reacts directly with CTR, with formation of argininesuccinic acid; enzymic cleavage of the latter leads to formation of arginine and then urea. It follows from this that for conversion of CTR to arginine (urea) the nitrogen of other amino acids must previously be transferred by one or another pathway to AS. As noted by Braunstein [4], a considerable part of protein nitrogen (up to 50%) can be transferred to urea in the second stage of the ornithine cycle without intermediate formation of ammonia, as a result of transamination reactions; in recent years it has been established that all or nearly all natural amino acids are susceptible to transamination. Another possible pathway of conversion of interest to us consists in deamination of amino acids with subsequent utilization of the liberated ammonia for synthesis of AS.

The extent of participation in this latter case has not yet been fully established of direct deamination and amination reactions, on the one hand, and of indirect conversions, mediated through transamination, that is, of processes of transdeamination and transreamination (according to the terminology of Braunstein [5]) – on the other hand. It has also not been explained whether AS formation occurs in liver from monocarboxylic L-amino acids by way of direct transamination between these amino acids and oxaloacetic acid (OA) or by way or a 2-step transfer of the NH_2-group – first to ketoglutaric acid (KG), and then from the L-glutamic acid (GL) formed to OA, that is, by way of 2-step transamination.

In this paper are presented results of studies, directed toward determination of the role of transamination reactions in the utilization of nitrogen of ammonia and of various amino acids in the second stage of urea formation, and also of the significance of KG (GL) as intermediary nitrogen carrier in this conversion.

EXPERIMENTAL METHOD

To solve these problems the formation of urea (and of ammonia) was studied in rat liver homogenates in the presence of DL-CTR, cell respiration substrates and ATP. Various N-donors were added in concentrations slightly exceeding the physiological amino acid content in tissue, i. e., in the amount of 20 micromoles per sample of 3 ml ($7 \cdot 10^{-3}$ M). In most experiments, during homogenization of liver, for the purpose of stabilization of the homogenate there was added the disodium salt of ethylenediaminetetraacetic acid (EDTA) with a final concentration in samples of $1 \cdot 10^{-3}$ M. For description of the experimental setup and analytical methods see reference [1].

In the first series of experiments urea formation was studied in liver homogenates of rats with vitamin B_6 deficiency and of control rats. The animals were kept on the synthetic diet used in our laboratory and were used for experiment only when marked signs of avitaminosis (acrodynia, excretion of large amounts of xanthurenic

acid) were present. For suppression of vitamin B_6 synthesis by intestinal microorganisms 2% phthalazol was added to the diet of some of the rats.

In other experiments in order to analyze the mechanism of conversion of various N-donors in liver homogenate we used the inhibition by selective enzyme poisons of individual intermediate reactions of cell metabolism, as well as exclusion from the composition of experimental samples of one or another component, necessary for urea synthesis (see below).

All values given in tables are averages of data from 3-6 (or more) experiments in good agreement, with normal intensity of urea synthesis in the complete experimental samples.

EXPERIMENTAL DATA

Urea Formation from Citrulline and Amino Acids in Liver Homogenates of Vitamin B_6-Deficient Rats

To solve the problem of the role of transamination reactions in the second stage of urea synthesis we tried to make use of liver homogenates of vitamin B_6-deficient rats, in whose tissues the activity of aminopherases is reduced. According to data of Berezov [6], in vitamin B_6-deficient rats in vivo there is a disturbance of urea formation from dietary protein and from certain L-amino acids. Proceeding on the assumption of participation of transamination reactions in formation of AS, it might be expected that in vitamin B_6 deficiency conversion of CTR to urea would be disturbed in liver homogenate during utilization of all amino acids, with the exception of AS.

It was found, however, that intensity of urea formation from CTR and GL is the same in normal and in vitamin B_6-deficient rats, as well as in experiments with L-alanine (AL) and with other N-donors.

It should be pointed out that in preliminary observations in vivo on the vitamin-deficient rats we employed, and also in experiments of other laboratory workers on animals with far advanced vitamin B_6 deficiency, it was not possible to establish at all regularly the decrease noted by Berezov of the percentage urea content in urine of the rats (see also studies of McHenry et al. [7, 8]). Direct study of the transamination reaction AL $\rightleftharpoons$ GL in liver homogenates showed that decrease in activity of this reaction, estimated by increase of intensity of the GL spot on chromatograms of incubated samples, was relatively small in our vitamin-deficient rats. Thus, it appears, that liver of vitamin B_6-deficient rats is not a suitable experimental subject for determining the role of transamination reactions in urea synthesis.

Metabolism of Amino Acids and Ammonia in Liver Homogenate During Inhibition of Transamination or of the Second Stage of Urea Synthesis

In further experiments to determine the role of transamination reactions in dissimilation of amino acid nitrogen in liver we used inhibition of transamination reactions by reagents for the carbonyl group, which bind phosphopyridoxal.

The action was tested of the following CO-reagents: hydroxylamine ($NH_2OH \cdot HCl$), semicarbazide, isonicotinylhydrazide (tubazid), phenylhydrazine and aminoguanidine; the most suitable for our purposes was NH_2OH.

By means of paper chromatography, in agreement with data of Braunstein, Azarkh and Mogilevskaya [9], we established that at the low substrate concentrations ($7 \cdot 10^{-3}$ M) in our experiments hydroxylamine (10^{-3} M) strongly inhibits the transamination reaction AL $\rightleftharpoons$ GL in liver homogenate. With addition of NH_2OH ($5 \cdot 10^{-4}$ to 10^{-3} M) there is observed in the homogenate a profound inhibition of urea synthesis from CTR and GL or monocarboxylic amino acids, while urea formation from CTR as AS is not disturbed.

An analogous, but somewhat less active effect, is shown by tubazid; the other carbonyl reagents act more weakly and inconstantly. In view of the fact that tubazid and other hydrazine derivatives are split in tissue homogenate with formation of ammonia, which obscures the picture of the conversions investigated, in further experiments only NH_2OH was used.

In the search for suitable agents for suppression of transamination, we tested, among other compounds, α-methylamino acids [9] and found that α-methyl-GL and α-methyl-AL do not inhibit the second stage of urea synthesis, while α-methyl-AS ($7 \cdot 10^{-2}$ M) under the conditions of our experiments almost completely suppresses urea formation from CTR and all N-donors, including AS (see Table 2, samples 8 and 10). Methyl-AS does not inhibit transamination reactions. It seemed probable that methyl-AS, acting as an antimetabolite of AS, inhibits synthesis of argininesuccinic acid from CTR and AS in "Ratner's reaction." This assumption was confirmed in a more detailed study of the mechanism of the effect of methyl-AS on the reaction of the ornithine cycle. We have used methyl-AS in a study of nitrogen dissimilation of various amino acids in liver homogenate in blockage of urea synthesis.

Besides addition of NH_2OH or methyl-AS to block urea synthesis, in a number of experiments we used omission from the composition of the incubated samples of individual components necessary for this process, for example CTR or respiratory substrate. We also used reagents which specifically inhibit certain enzymes participating in the metabolism of individual amino acids, for example benzoic acid, EDTA and others.

In the composite Tables 1 and 2 are given the results obtained by the methods indicated in the study of conversion of nitrogen of various amino acids by rat liver homogenates to urea nitrogen and ammonia.

In examining these tables the attention is first of all struck by the fact that addition of NH_2OH inhibits urea formation from CTR and all amino acids, except AS (Tables 1 and 2, Sample 9). Inhibited also is increase of urea in samples with CTR without addition of N-donor (Sample 6); as noted previously [1], under these conditions donors (NH_3 and amino acids) preformed in liver tissue serve in part as sources of the second nitrogen atom of urea, in part ornithine, formed in the cycle of urea formation from CTR.

These data indicate that conversion of nitrogen of amino acids (except AS) to urea depends on reactions which are inhibited by NH_2OH. Absence of inhibition in experiments with AS indicates that transamination reactions are inhibited, which are necessary for conversion of nitrogen of other amino acids to AS.

Hydroxylamine suppresses also utilization of nitrogen of salts of ammonia for urea synthesis from CTR (Table 1, Samples 9 and 8); to the decrease, on the average of 7.7 micromoles, in the urea rise there is a corresponding parallel increase of residual NH_3 content of 7.3 micromoles in samples incubated with NH_2OH. Evidently also in utilization of ammonia in the second stage of urea synthesis there is, as a necessary stage, a conversion, suppressed by NH_2OH, which apparently is transamination.

In experiments where D-amino acids (AL and phenylalanine (PhAL) serve as N-donors, and likewise L-histidine, inhibitions of urea synthesis by hydroxylamine (Sample 9) or methyl-AS (Sample 10), as well as inhibition of urea synthesis by omission from samples of respiratory substrate (Sample 13) or CTR (Samples 3 and 4), leads to ammonia accumulation. The amount of excess ammonia is approximately equivalent to the decrease of the urea rise in comparison with the corresponding complete samples (Sample 9). Experiments with use of benzoic acid as specific inhibitor, suppressing the action of D-amino acid oxidase and glycine oxidase, showed that urea formation from D-AS and D-PhAL is abolished in thr presence of benzoic acid, and ammonia accumulation does not occur. At the same time benzoic acid does not influence urea synthesis from CTR and NH_4Cl and consequently, does not exert a non-specific toxic effect on the homogenate. All these experiments indicate that nitrogen of D-amino acids is utilized in the second stage of urea synthesis after its preliminary cleavage in the form of NH_3, as a result of deamination of these amino acids by D-amino acid oxidase.

In contrast to this, in experiments with most of the amino acids of the L-series (CTR, AS, GL, AL, PhAL, proline, lysin) and glycine, in those samples where urea synthesis is suppressed by any of the methods indicated (in samples with NH_2OH or methyl-AS, without CTR or fumarate), the ammonia content is not higher than the ammonia content in samples of corresponding composition, but not containing the indicated amino acids. It is evident that these amino acids, introduced into samples in concentrations close to physiological, are not deaminated by liver tissue, although under the same experimental conditions they are utilized effectively as N-donors for conversion of CTR to urea.

Conversion of nitrogen of these amino acids to AS and further to urea occurs through transamination reactions, without intermediate ammonia formation.

TABLE 1

Utilization of Nitrogen of L- and D-Amino Acids with NH_4Cl in Urea Formation from Citrulline in Liver Homogenates Under Various Conditions (Average Values) Composition of Samples: 0.5 ml homogenate (1:3); DL-CTR – 40 μM; ATP – 5 μM; $MgSO_4$ – 10 μM; fumarate – 20 μM; N-donors: amino acids – 20 μM; NH_4Cl – 15 μM. Concentrations: EDTA (in all samples) – 10^{-3} M; NH_2OH – 10^{-3} M; methyl-AS – $7 \cdot 10^{-2}$ M; benzoic acid – $5 \cdot 10^{-4}$ M. Incubation – 1 hr. at 38° in O_2 atmosphere. Urea and NH_3 contents are given in micromoles in sample after incubation.

No. of sample	Supplements to samples	N-Donors															
		L–AS		L–GL		L-proline		NH_4Cl		L–AL		D–AL		L–PhAL		D–PhAL	
		urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3
1	Without supplement (homogenate + ATP + $MgSO_4$+EDTA	—	—	—	—	—	—	—	—	0.5	6.8	0.5	6.8	1.0	9.5	1.0	9.5
2	Fumarate	—	—	—	—	—	—	—	—	0.5	4.5	0.5	4.5	—	—	—	—
3	N-donor	—	—	—	—	—	—	—	—	0.4	7.1	1.0	12.1	1.2	9.2	1.0	15.0
4	N-donor; fumarate	—	—	—	—	—	—	—	—	0.5	3.0	0.6	7.2	—	—	—	—
5	CTR; fumarate	5.8	3.3	5.8	3.3	3.2	4.9	3.3	3.6	5.6	3.4	5.6	3.4	3.0	4.3	3.0	4.3
6	CTR; fumarate; NH_2OH	3.3	5.3	3.3	5.3	2.0	6.6	2.3	4.6	4.0	5.5	4.0	5.5	2.1	5.8	2.1	5.8
7	CTR; fumarate; methyl-AS	2.0	4.3	2.0	4.3	—	—	—	—	2.0	4.3	2.0	4.3	1.0	5.0	1.0	5.0
8	CTR; fumarate; N-donor	15.6	2.4	14.5	2.4	14.0	1.0	11.2	10.0	14.8	4.3	12.0	2.7	5.8	5.3	7.7	9.3
9	CTR; fumarate; N-donor; NH_2OH	14.7	3.8	5.5	3.8	2.7	5.2	3.5	17.3	4.9	4.3	1.4	9.0	1.1	7.5	1.0	15.0
10	CTR; fumarate; N-donor; methyl-AS	5.3	3.8	4.9	4.0	—	—	—	—	2.7	3.4	2.0	10.0	1.2	8.4	1.0	15.0
11	CTR; fumarate; N-donor; benzoic acid	—	—	—	—	—	—	9.8	10.8	—	—	5.4	4.6	—	—	3.4	7.4
12	CTR; fumarate; benzoic acid	—	—	—	—	—	—	3.0	4.2	3.2	4.0	3.2	4.0	—	—	—	—
13	CTR; N-donor	3.8	4.0	11.7	2.5	—	—	—	—	4.6	6.6	1.4	9.3	—	—	—	—

TABLE 2

Utilization of Nitrogen of Certain Amino Acids in Urea Formation from Citrulline in Liver Homogenates under Various Conditions.
For experimental conditions see Table 1.

No. of sample	Supplements to samples	N-Donors									
		Glycine		L-lysine		L-serine		D-serine		L-histidine	
		urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3
1	Without supplement (homogenate+ATP+$MgSO_4$+EDTA*	—	—	1.0	0.5	1.4	9.5	1.4	9.5	0.9	3.2
3	N-Donor	—	—	—	—	1.4	14.3	1.0	9.5	1.0	13.7
5	CTR; fumarate	5.2	3.0	2.6	5.0	11.0	2.8	11.0	2.8	6.6	3.3
6	CTR; fumarate; NH_2OH	2.7	4.7	1.5	6.8	—	—	—	—	4.3	4.3
8	CTR; fumarate; N-donor	10.0	1.0	10.4	1.4	18.0	2.8	7.7	7.4	11.0	6.0
9	CTR; fumarate; N-donor; NH_2OH	2.6	5.0	2.7	5.4	1.0	10.0	1.0	9.0	4.8	11.8
11	CTR; fumarate; N-donor; benzoic acid	9.3	1.3	—	—	—	—	—	—	—	—
12	CTR; fumarate; benzoic acid	4.1	3.6	—	—	—	—	—	—	—	—
13	CTR; N-donor	—	—	—	—	—	—	—	—	2.5	12.9

*In experiments with histidine the homogenate was prepared without addition of EDTA.

The L- and D-isomers of serine behave somewhat differently. From D-serine neither urea nor ammonia is formed in the liver. As to L-serine, it is anN-donor in the second stage of urea synthesis (Table 2); in the absence of CTR (Sample 3) ammonia is formed from L-serine. In the sample with CTR, fumarate and NH_2OH, not only is urea formation suppressed, but also ammonia formation from L-serine (compare Samples 1, 3 and 8 Table 2). This is linked with the fact that L-serine is deaminated in a nonoxidative reaction by the phosphopyridoxal enzyme (serine dehydrase), whose action is inhibited by hydroxylamine.

In experiments with glycine, benzoic acid does not influence urea formation, in contrast to NH_2OH, in the presence of which conversion of glycine to urea is suppressed (Samples 6 and 9, Table 2); deamination of glycine is not observed in a single incubated sample. The data presented make it possible to exclude participation of glycine oxidase in glycine dissimilation in liver; they indicate that glycine nitrogen is converted to urea through transamination reactions without intermediate ammonia formation.

The ammonia formation noted above from L-histidine during inhibition of urea synthesis occurs as a result of anaerobic cleavage of this amino acid by a specific enzyme ("histidase" or histidine deaminase). However, as indicated by the experimental results, shown graphically in Figure 1, L-histidine nitrogen can be transferred to urea not only after its splitting off by histidine deaminase, but also without conversion to ammonia. The action of histidine deaminase can be completely excluded by EDTA (10^{-4} M), which is a specific inhibitor of this enzyme [10].

As is evident from the data shown in Figure 1 (Samples II, III, VIII) ammonia is formed in liver homogenate from histidine, if CTR or respiratory substrate is absent from the system, or if NH_2OH is added. Ammonia formation does not occur in samples in which EDTA is simultaneously added (Samples IV, X); this indicates that deamination was produced by histidine deaminase. In the complete system in the presence of L-histidine there is formed on the average 11 micromoles of urea (Sample VII); with addition of NH_2OH urea synthesis is reduced to 4.8 micromoles (almost as much urea is formed in the corresponding sample V, not containing histidine), and the amount of NH_3 is increased from 6 to 11.8 micromoles (compare samples VIII and VII). At the same time in sample IX, where histidase deamination of L-histidine is completely suppressed by EDTA, urea synthesis

proceeds at the same level as in the complete Sample VII without EDTA. With simultaneous addition of EDTA and NH_2OH, that is with inhibition of both histidase deamination and also transamination, there is formed from L-histidine neither urea nor ammonia (Sample X, compare with Sample V). These experiments indicate that histidase deamination with subsequent utilization of ammonia, on the one hand, and transamination – on the other, represent two alternative steps on the pathway of conversion to urea of the amino group nitrogen of histidine in the second stage of the ornithine cycle.

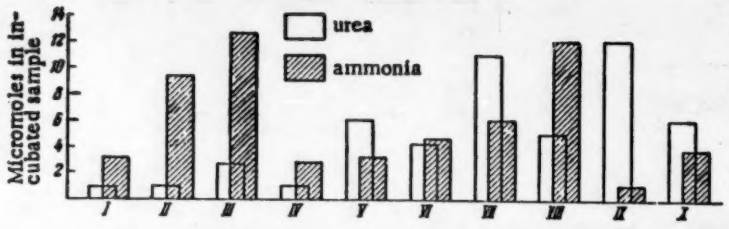

Fig. 1. Formation of urea and ammonia from L-histidine in liver homogenate. Samples contained the homogenate, ATP, $MgSO_4$ and the supplements: I) fumarate; II) fumarate, histidine; III) CTR, histidine; IV) fumarate, histidine, EDTA; V) CTR, fumarate; VI) CTR, fumarate, NH_2OH; VII) CTR, fumarate, histidine; VIII) CTR, fumarate, histidine, NH_2OH; IX) CTR, fumarate, histidine, EDTA; X) CTR, fumarate, histidine, EDTA, NH_2OH.

Role of α-Ketoglutaric Acid in the Second Stage of Urea Synthesis in Amino Acid Dissimilation in Liver Tissue

It was shown above that NH_2OH inhibits urea synthesis from CTR and ammonia, added to the homogenate in the form of NH_4Cl or formed as a result of oxidative deamination of D-amino acids (or nonoxidative deamination of certain L-amino acids, for example L-histidine). These observations indicate that ammonia nitrogen in the process investigated can not be transferred to the NH_2-group of AS as the result of direct amination of OA; most probably there is an indirect mechanism of amination of OA, which consists in amination of KG by glutamic dehydrogenase with subsequent transamination between GL and OA ("transreamination" [5]).

With respect to transfer of nitrogen of natural monocarboxylic amino acids to AS without intermediate ammonia formation the question also arises, whether this transfer proceeds by direct, single-step transamination between these donors and OA, or whether a 2-step transamination is necessary with participation of KG as intermediate acceptor of the NH_2-group. If the conversion KG →GL is an obligatory link in conversion of nitrogen of ammonia and of monocarboxylic acids to AS, then under conditions which exclude the possibility of KG formation, there should be in liver homogenate an inhibition of urea formation from CTR and all N-donors, with the exception of L-AS and L-GL (and also proline, which is readily converted by oxidation to GL). The inhibition of urea synthesis produced should be overcome by addition of KG to the incubated samples.

In order to exclude from the experimental system KG, which is readily formed in reactions of the citric acid cycle from respiratory substrate (fumarate) and from certain amino acids, at the suggestion of Professor A. E. Braunstein, we made use of the possibility of interrupting the citric acid cycle at the stage which precedes KG formation, by means of fluoroacetic acid [5].

Fluoroacetic acid, entering the citric acid cycle, is converted to fluorocitric acid, which strongly inhibits the action of aconitase and thus prevents KG formation from citric acid and its precursors [11].

Sodium fluoroacetate (FA) was added to samples with liver homogenate containing EDTA (final concentration of FA $5 \cdot 10^{-3}$ M). Samples with FA and without it (controls) were incubated 15 minutes in an atmosphere of oxygen in the presence of fumarate, ATP and $MgSO_4$, in order to assure formation of fluorocitric acid. Then the

TABLE 3

Effect of Fluoroacetate ($5 \cdot 10^{-3}$ M) on Conversion of Citrulline to Urea in Liver Homogenate. Experimental conditions – see Table 1; all samples contained, in addition to supplements indicated in the table, ATP, $MgSO_4$ and EDTA

No. of sample	Supplements to sample	N-Donors									
		L–AS		L–GL		NH_4Cl		L–AL		D–AL	
		urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3	urea	NH_3
1	CTR; fumarate; N-donor	14.3	1.6	17.6	1.2	16.2	5.7	15.0	7.2	12.6	4.2
2	CTR; fumarate; N-donor; FA	3.9	7.3	16.8	3.4	1.8	18.2	5.4	6.5	4.9	12.9
3	CTR; fumarate; N-donor; phosphoglyceric acid	14.5	1 0	—	—	—	—	—	—	—	—
4	CTR; fumarate; N-donor; phosphoglyceric acid	4.1	6 4	—	—	2.5	15.0	—	—	—	—
5	CTR; fumarate; N-donor; KG; FA	10.7	7.5	—	—	14.6	6.2	—	—	—	—

vessels were immersed in ice, substrates (CTR, N-donors, etc.) were added, they were saturated with O_2, and incubation was continued for an hour.

The results of these experiments, given in Table 3, show that urea formation from CTR and GL, as might be expected, is not reduced in the presence of FA (Samples 1 and 2). With use of fumarate as respiratory substrate FA produces complete inhibition of urea formation from NH_4Cl, L-AL, D-AL and – unexpectedly – also from L-AS. And where NH_4Cl or D-AL was utilized in the samples incubated with FA, there is observed an increase of ammonia content, equivalent to the decrease of urea synthesis. With introduction of KG into samples in addition to fumarate (Sample 5) or instead of fumarate (not shown in table) the inhibiting action of FA is decreased. The unforeseen inhibition of urea formation from AS by means of FA can not be attributed to disturbance of transamination reactions due to absence of KG, since AS reacts with CTR directly. It can be assumed that this inhibition is caused by disturbance of respiratory resynthesis of ATP (necessary for synthesis of arginine succinic acid) as the result of blocking by FA of oxidation of respiratory substrate at the citric acid stage. An attempt to assure ATP resynthesis under these conditions by introduction into samples of an additional source of high-energy phosphate residues in the form of phosphoglyceric acid (20 micromoles) and thus remove the inhibiting action of FA in experiments with AS was not successful (Samples 3, 4, 5). However, it must be taken into account that, as Ratner [2] notes, phosphoglyceric acid under the given experimental conditions acts chiefly as a source of pyruvic acid, and not as donor of energy-rich phosphate groups.

The inhibiting action of FA on urea formation from CTR and AS does not permit any definite conclusions to be made from these experiments concerning the role of KG as carrier of the NH_2-group in transfer to L-AS of nitrogen of ammonia and various amino acids.

In order to reach a solution of this problem, a series of additional experiments was done, in which dissimilation of certain amino acids, i. e., L-AL, D-AL and glycine, in liver homogenate under various conditions was studied by measurement of the decrease of these amino acids by specific photometric methods [1, 2], parallel with determination of urea and ammonia. Homogenate samples (stabilized with ATP, $MgSO_4$ and EDTA) were incubated, containing one amino acid, in the presence of fumarate, of fumarate and CTR (complete system of urea synthesis), and also samples to which, in addition, was added FA without KG or with KG. Results of experiments of this type are given graphically in Figure 2, where in the form of columns is shown the decrease of amino acid in percent (its content in the sample, analyzed before incubation, is taken as 100%).

From this diagram it is evident that L-AL, added to liver homogenate without fumarate and CTR (I), does not disappear to any extent during incubation for 1 hour; the small decrease of L-AL must be attributed to transamination with endogenous keto acids. Addition of fumarate leads to considerable decrease of L-AL without increase of urea or NH_3, evidently attributable to transamination with keto acids, arising during fumarate oxidation (Sample II). This decrease of L-AL is reduced to less than half, if together with fumarate FA (III) is added.

With addition of KG to the sample with FA and fumarate the decrease of L-AL again increases (IV). In the sample where the presence of all necessary cofactors, fumarate and CTR assures urea synthesis (V) 98% of the L-AL disappears; NH_2OH under these conditions completely suppresses dissimilation of L-AL (VI).

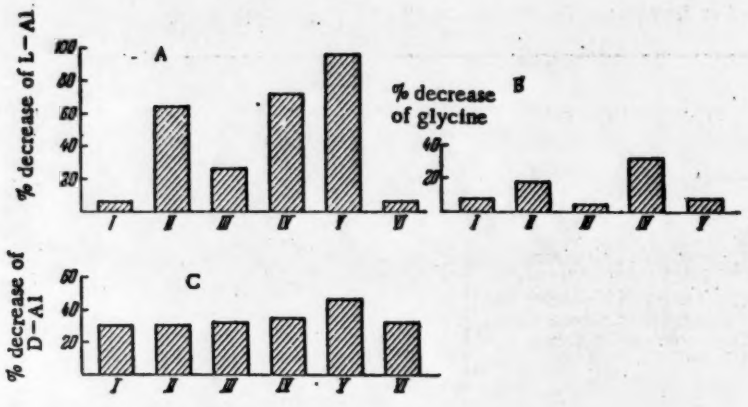

Fig. 2. Dissimilation of L-AL, D-AL and glycine in rat liver homogenate
The content of the corresponding amino acid in the unincubated sample is taken as 100%: I) without supplements; II) fumarate added; III) fumarate and FA; IV) fumarate, FA and KG (A); CTR and fumarate (B); CTR (C); V) CTR, fumarate (A and C); CTR, fumarate, NH_2OH (B); VI) CTR, fumarate, NH_2OH.

Analogous results were obtained in experiments with glycine. With addition to the homogenate of glycine alone its dissimilation during the experimental period is insignificant (I). In the sample with fumarate the glycine decrease reaches 18% (II), while this decrease is reduced in the presence of FA (III). In the sample with fumarate and CTR (IV) glycine decrease reaches 32%; hydroxylamine under these conditions inhibits the glycine decrease nearly completely (V).

In identical experiments with D-AL there occurs disappearance of nearly the same amount of AL in all samples (I – VI), while in the complete sample with CTR and fumarate (V), urea is formed, and in the others – ammonia. Neither the presence of respiratory substrate (II), CTR (IV), NH_2OH (VI) nor addition of FA (III) influences the magnitude of the D-AL decrease. Alanine decrease is somewhat increased only in sample V.

DISCUSSION OF RESULTS

To determine the role of transamination reactions in conversion to urea of the nitrogen of various amino acids in the first series of experiments we attempted to utilize liver tissue of rats with vitamin B_6 deficiency, on the basis of the participation of phosphopyridoxal in the action of aminopherases, and of the data of Berezov [6] on disturbance of urea formation in vitamin B_6-deficient rats.

However, we did not observe in liver homogenates of rats with marked vitamin B_6 deficiency either decrease of urea synthesis from amino acids and citrulline, or a significant decrease of activity of aminopherases (in a study by the method of paper chromatography). These results are in agreement with the data of McHenry and coworkers [7, 8] on the absence of disturbances of urea formation in vivo and in liver slices in vitamin B_6-deficient animals. Comparison of our data with those in the literature on the residual activity of liver aminopherases in vitamin B_6 deficiency with physiological values of urea formation shows that even in profound vitamin B_6 deficiency disturbance of transamination processes does not reach such a degree as to be reflected in urea synthesis. According to the data of McHenry et al., transamination values in liver, expressed in the so-called Warburg coefficients ($Q_{transamination}$) are reduced in vitamin B_6 deficiency in the reaction GL $\rightleftharpoons$ AS with an average Q = 350 (in the normal) to Q = 170, and in the reaction GL $\rightleftharpoons$ AL with Q = 270 to Q = 98. It is evident

that the activity of aminopherases which has been retained is amply sufficient to assure urea synthesis, the intensity of which in liver slices, according to data of the same authors, is expressed by values (Qurea formation) of 7-8.

Application of a number of methods which disturb urea formation in liver homogenates of normal rats provided an opportunity to make definite conclusions with respect to the mechanism of dissimilation of various amino acids in liver and with respect to the pathways of utilization of this nitrogen in the second stage of urea synthesis. Inhibition of transamination reactions by hydroxylamine or by other carbonyl poisons suppresses urea formation from CTR at the expense of the nitrogen of ammonia and all amino acids tested, except AS. It follows from this, that transamination reactions participate in transfer of the nitrogen of all these donors to AS. During blockage of synthesis of argininesuccinic acid ("Ratner's reaction") by an antimetabolite of AS – α-methylaspartic acid, and also during omission from the system of respiratory substrate or CTR, there is disturbance of urea formation from all N-donors, including AS. In experiments with D-amino acids inhibition of urea synthesis by any of the methods mentioned is accompanied by accumulation in the samples of an equivalent amount of ammonia; the greatest ammonia accumulation is observed in samples without respiratory substrate, where the possibility is excluded not only of urea synthesis, but also of utilization of NH_3 for amination of keto acids, arising in the citric acid cycle. Thus in disturbed urea synthesis the deamination of D-amino acids is apparent. This represents a necessary link in dissimilation of D-amino acids in liver and in conversion of their nitrogen to urea; during inhibition of Krebs' D-amino acid oxidase by benzoic acid not only is urea synthesis from D-amino acids completely abolished, but also their deamination.

In experiments with L-amino acids the inhibition of urea synthesis is not accompanied (for a few exceptions, see below) by ammonia formation. Our experimental data lead to the conclusion that in physiological concentrations ($7 \cdot 10^{-3}$ M) glycine and the L-isomers of CTR, AS, GL, AL, PhAL, proline, lysine (and other amino acids) are practically not deaminated in liver homogenates. At the same time their nitrogen is readily converted under the same conditions to urea without intermediary ammonia formation, being transferred as a result of transamination reactions to AS, which further reacts with CTR in the second stage of the ornithine cycle.

By this pathway in liver homogenate are dissimilated rapidly and in considerable quantities amino acids, such as lysine and glycine, whose breakdown, according to the data in the literature, in surviving mammalian liver tissue under other conditions proceeds very slowly and is usually detected only by the isotope method.

Direct oxidative deamination of GL and transdeamination of certain other amino acids with participation of GL dehydrogenase [5, 13] occur to an appreciable extent only at high substrate concentrations and at alkaline pH values. Under more physiological conditions these processes, apparently, do not play an essential part in nitrogen dissimilation of amino acids in liver tissue since, under these conditions, the action of GL dehydrogenase is chiefly in the direction of GL synthesis. Nor does oxidative deamination take part in glycine dissimilation in liver; this is confirmed by the fact that benzoic acid, which inhibits the action of glycine oxidase, does not disturb urea formation from glycine.

The fact that dissimilation of natural amino acids in liver actually proceeds chiefly by way of transamination and conversion to urea through AS has been shown, with L-AL and glycine as examples, by experiments, in which parallel with determination of formation of NH_3 and urea the decrease of these amino acids (Figure 2) was measured directly. Disappearance of L-AL and glycine occurs on an appreciable scale only when the possibility of transamination is assured (in the presence of fumarate), and it is significantly increased when the irreversible conversion of AS to urea prevents setting up an equilibrium in the reversible reactions of transamination (that is, with addition of CTR). Inhibition of decrease of L-AL and glycine by FA and removal of this inhibition by addition of KG indicate that in dissimilation of these amino acids (and probably also in other cases) there is no direct transfer of the NH_2-group to OA, but a 2-step transamination with participation of KG (GL). FA inhibits urea formation from CTR and amino acids (except GL), but this does not permit a definite conclusion to be made concerning the obligatory participation of KG in nitrogen transfer under the given conditions, since FA also suppresses urea synthesis in the homogenate from AS and CTR.

In urea formation from certain natural amino acids, besides transamination reactions, specific mechanisms of anaerobic deamination play a definite part, as is shown by the results of our experiments with L-histidine and L-serine. During inhibition by hydroxylamine of transamination and urea synthesis from L-histidine, as a result of its cleavage by histidase (histidine deaminase), ammonia is formed. However, complete inhibition of histidase

deamination by EDTA does not disturb urea formation from L-histidine. Evidently nitrogen of the L-histidine amino group is utilized in the second stage of urea synthesis by two parallel and mutually replaceable pathways – with intermediary histidase deamination and through transamination reactions without splitting off of ammonia.

From L-serine in liver homogenate in the absence of CTR ammonia is formed. If urea synthesis is suppressed by NH_2OH, ammonia formation from serine is absent, since hydroxylamine inhibits, together with transamination, nonoxidative deamination of L-serine by serine dehydratase [5].

It is entirely probable, in dissimilation of the L-isomers of threonine, cysteine, cystathionine and methionine in liver and in nitrogen utilization in the second stage of urea formation, just as in the case of serine which we studied, that a part is played by nonoxidative cleavage of ammonia from these amino acids by the action of the pyridoxal enzymes [5]. Nor is conversion to urea without ammonia cleavage, through transamination reactions, excluded for certain of these amino acids, for example L-methionine and, perhaps, L-serine [14].

As we have seen, ammonia, formed as the result of nonoxidative cleavage of L-histidine or of oxidative deamination of D-amino acids, or added to the homogenate, in the presence of hydroxylamine, does not disappear and is not utilized for conversion of CTR to urea.

This fact, in agreement with the results of other studies carried out in our laboratory and with the data of Ratner [3], indicates that ammonia nitrogen is converted in the liver to AS not by means of direct amination of OA, but with participation of aminopherases, as a result of transreamination (Braunstein [5]). The KG necessary for this is formed in the citric acid cycle from added respiratory substrate (fumarate). In the work of Azarkh [13] it was shown by direct experiments, that FA, which inhibits KG formation in the citric acid cycle, suppresses utilization of NH_3 for amination of OA and pyruvic acid in liver tissue, but does not inhibit amination of KG.

It is evident from what has been said, that in breakdown of natural amino acids in mammalian liver oxidative deamination does not take an essential part. In their dissimilation the chief role is played by indirect conversions, mediated through transamination, in accordance with the concepts developed by A. E. Braunstein and coworkers.

Nitrogen of the majority of natural amino acids during their dissimilation in liver is transferred as a result of the transamination reaction to L-AS, which is then utilized in the second stage of the ornithine cycle as the only direct source of half of the urea nitrogen. Transfer of nitrogen of L-AL, glycine and other monocarboxylic amino acids to AS proceeds, apparently, in two steps, with intermediary formation of GL. Nitrogen of certain amino acids, which can undergo in liver direct nonoxidative cleavage with ammonia formation (histidine, serine and others), is also utilized in the second stage of urea formation only through participation of transamination reactions, inasmuch as conversion of ammonia to AS proceeds through GL, by means of transreamination.

From this it follows that nearly half of the nitrogen of proteins broken down in the organism, which is excreted in mammals in the form of urea, enters the structure of the latter without intermediary conversion to free ammonia.

A question arises concerning the origin of the ammonia, necessary for conversion of ornithine to citrulline in the first stage of urea synthesis. If it is taken into account that oxidative deamination of L-amino acids in liver is not a substantial source of ammonia, and that amino acids, susceptible to anaerobic deamination, represent only a small part of the protein molecule, then it must be admitted that the main mass of ammonia, which serves as the source of half the nitrogen of urea in the first stage of its synthesis, is formed outside the liver – as a result of deamination (direct and indirect) of amino acids in various organs and of breakdown of other nitrogen compounds. This ammonia must enter the liver with the bloodstream, chiefly in the form of the amide group of glutamine, which is hydrolyzed in liver by the corresponding enzyme systems.

SUMMARY

In liver homogenate of vitamin B_6-deficient rats urea formation from citrulline and various amino acids is not disturbed, which is explained by the high residual activity of aminopherases. Inhibition of transamination reactions by carbonyl reagents (NH_2OH, isonicotinylhydrazide) strongly suppresses urea synthesis from CTR and all N-donors (ammonia, amino acids) studied, except L-AS.

α-Methyl-DL-aspartic acid suppresses urea synthesis from CTR and from all N-donors, even including L-AS. Methyl-AS acts as an antimetabolite of AS in the condensation reaction of the latter with CTR.

During inhibition of urea synthesis (in the presence of NH_2OH or methyl-AS; in samples without CTR or respiratory substrate) liver homogenate forms ammonia from L-amino acids and does not form it from glycine and most D-amino acids.

Benzoic acid – specific inhibitor of the flavine oxidases of glycine and D-amino acids – does not inhibit glycine utilization in the second stage of urea synthesis, but suppresses formation of urea (and of ammonia) from D-amino acids.

Nonoxidative deamination may precede utilization of nitrogen of certain L-amino acids (e. g., histidine, serine) in the second stage of urea formation. Upon suppression by EDTA of histidase deamination the amino nitrogen of L-histidine is utilized in the second stage of the ornithine cycle through transamination reactions.

The total of the experimental results indicates that in liver tissue all N-donors react with CTR in the second stage of urea synthesis only after conversion of their nitrogen to L-AS. Most of the natural amino acids in physiological concentrations are not deaminated in liver homogenate; AS and, further, urea is formed from them in liver almost exclusively by way of transamination, and from ammonia – by means of transreamination, through GL. The D-isomers of amino acids first undergo oxidative deamination, and certain L-amino acids are in part deaminated in nonoxidative reactions.

In transfer of nitrogen of monocarboxylic acids to AS, judging by the data obtained upon inhibition by FA of KG formation, a significant, if not the principal, part is played by 2-step transamination reactions with participation of KG as intermediary carrier of NH_2-groups.

I am grateful to Professor A. E. Braunstein for the guidance of this work.

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MECHANISM OF DISTURBANCE OF TYROSINE OXIDATION IN THE LIVER IN PROTEIN DEFICIENCY

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In a previous study from our laboratory [1], it was established that one of the earliest manifestations of protein deficiency in animals is a marked disturbance of oxidation of tyrosine and phenylalanine in the liver. In discussion of the question of possible causes of this disturbance, we suggested that one of the factors producing it is an endogenous avitaminosis C, which in rats always accompanies protein deficiency [2].

Participation of ascorbic acid in the process of tyrosine oxidation in liver was established a relatively long time ago [3]. In recent years there has been obtained sufficiently convincing evidence of the fact that ascorbic acid is necessary for conversion of p-hydroxy-phenylpyruvic acid, which is formed in the first stage of tyrosine oxidation in liver by means of transamination with ketoglutaric acid, to 2,5-dihydroxyphenylpyruvic acid, which appears as the next intermediate product of tyrosine oxidation. In a study of Williams and Sreenivasan [5] data were further obtained which showed that ascorbic acid probably takes part in another step of tyrosine oxidation, namely at the stage of cleavage of homogentisic acid with formation of fumarylacetoacetic acid. In the same study a scheme of tyrosine oxidation is given, according to which a necessary cofactor in this process is glutathione as well, which takes part, according to the data of the authors, in the reaction of conversion of 2,5-dihydroxy-phenylpyruvic acid to homogentisic acid. In recent work of Knox and Edwards [6] the data with respect to participation of glutathione in the process of tyrosine oxidation were confirmed, but these authors showed that glutathione is necessary, not for conversion of dihydroxyphenylpyruvic acid to homogentisic acid, but participates in the reaction of oxidation of homogentisic acid, acting as an activator of the specific oxidase for this acid.

Data concerning the role of glutathione in the process of tyrosine oxidation permit the suggestion that disturbance of tyrosine oxidation in protein deficiency may be a consequence of the decreased amount of reduced glutathione in liver, inasmuch as in the work of Shamshikova and Sinitsina-Ioffe [7] it was shown that glutathione synthesis in liver in protein deficiency is markedly lowered or completely abolished. It has also been impossible to exclude the suggestion that the primary cause of disturbance of tyrosine oxidation in protein deficiency is disturbance of one or several proteins – components of enzyme systems, participating in the complex process of formation of the final products of tyrosine metabolism – acetoacetic acid and fumaric acid. Inasmuch as decrease of tyrosine oxidation is a constant accompaniment of nutritional disturbances in animals and man and of the associated disorders of liver function, the clarification of the mechanism of this disturbance was of a certain theoretical and practical interest. The data obtained on this question are presented below.

EXPERIMENTAL METHODS

The experiments were done on white rats, 120-150 g, maintained on a low-protein diet (3% protein), the composition of which has been described previously [8]. When marked symptoms of protein deficiency appeared in the animals and the protein content of the blood serum fell below 5%, they were killed and slices or homogenates were prepared from the liver in the usual way. Liver preparations were incubated in Ringer-bicarbonate

* Deceased.

buffer (pH 7.4) for 2 hours at 38° with tyrosine (2 mg per 200 mg of tissue), after which the unoxidized tyrosine was determined. Determination of tyrosine in most experiments was done by the method of Udenfriend and Cooper [9], which is more sensitive than the method of Arnow which we used previously. The amounts of ascorbic acid, glutathione and other cofactors added to the incubation mixture varied in different experiments.

EXPERIMENTAL RESULTS

The data on the influence of ascorbic acid and glutathione on tyrosine oxidation by liver slices and homogenates of normal rats and rats with protein deficiency are given in Table 1.

TABLE 1

Effect of Ascorbic Acid and Glutathione on Tyrosine Oxidation.
Weight of slices – 200 mg; homogenate – 20 or 40%; amount of added ascorbic acid – 1 mg, glutathione – 1.5 mg

Liver preparation	Tyroxine oxidation in % of initial					
	without addition of cofactors		with addition of ascorbic acid		with addition of glutathione	
	K	O	K	O	K	O
20% Homogenate	25	9	31	12	—	—
"	27	11.5	26	10	26	11
"	25	8.5	29	8.5	27	7
40% Homogenate	39	6.5	37	7	37	6
"	34	11	—	15	34	11
"	42	13	40	15	40	13
Slices	45	4	49	7	47	6
"	44	8	50	12	46	11
"	35	12	34	14	38	10
"	48	18	48	22	47	21
"	46	0	46	3	46	0
"	42	18	47	21	44	20

K – normal rats.
O – rats with protein deficiency.

As is evident from the data presented, tyrosine oxidation in liver slices of normal rats varied within the limits 40-50% of the initial amount, which corresponds completely with the data we obtained previously. Tyrosine oxidation in liver homogenates occurred with somewhat less intensity and varied within the limits 25-40%. Neither addition of ascorbic acid nor addition of glutathione showed a perceptible effect on intensity of tyrosine oxidation in liver preparations of normal rats. This, of course, might be a result of the fact that in liver of normal animals the content of these compounds is sufficient for the process of tyrosine oxidation to proceed with maximal velocity under the given conditions.

Tyrosine oxidation in liver of rats on a low-protein diet in nearly all the experiments was 3-4 times lower than in normal rat liver. It should be noted that protein deficiency in all animals of this series was very marked and protein content of serum did not exceed 4.5%. Determinations of ascorbic acid in liver of these animals carried out in certain experiments showed that its amount was also sharply reduced and was only 30-35% of normal. Addition of ascorbic acid and glutathione to liver tissue of the experimental rats in certain cases showed an effect on intensity of tyrosine oxidation, but this effect was seldom observed and was evident to only an insignificant extent. This, of course, does not mean that deficiency of ascorbic acid or glutathione has no effect on tyrosine oxidation in liver of animals on a low-protein diet, but indicates only that under the given conditions tyrosine oxidation is limited by some other factor, also necessary for the normal course of the process.

To determine this factor we decided to follow the intensity of the individual reactions, the total of which leads to formation of the final products of tyrosine oxidation. As is known, the first reaction in this sequence is transamination of tyrosine with ketoglutaric acid. We have shown previously [8] that various enzyme systems of transamination are disturbed to a considerable degree in dietary protein deficiency. This provided a basis for the assumption that in fact the disturbance of the transamination reaction of tyrosine with ketoglutaric acid is the cause of the sharp decrease in intensity of tyrosine oxidation in liver of animals on a low-protein diet.

To clarify this problem special experiments were carried out, in which to liver slices and homogenates, during incubation with tyrosine, besides ascorbic acid and glutathione, ketoglutaric acid was also added and thus favorable conditions were created for the transamination reaction of tyrosine. The results of these experiments are given in Table 2.

TABLE 2

Effect of Ketoglutaric Acid on Tyrosine Oxidation in Liver Slices and Homogenates of Rats on a Low-Protein Diet.

Weight of slices – 250 mg; amount of added ketoglutaric acid – 2 mg, ascorbic acid – 1 mg, glutathione – 1.5 mg

Liver preparation	Tyrosine oxidation in % of initial			Liver preparation	Tyrosine oxidation in % of initial		
	in absence of cofactors	with ketoglutaric acid	with ketoglutaric acid and ascorbic acids		in absence of cofactors	with ketoglutaric acid	with ketoglutaric acid and ascorbic acids
Slices	13.0	37.8	47	Slices	14.3	37.5	58
"	6.1	25.5	39	"	2 5	18.0	32
"	8.3	41.1	46	"	8.9	24.5	25
"	12.98	35.0	48	"	11.3	19 7	34
"	7.7	22.5	40	"	10.4	28.7	39
"	12.1	25.5	45	"	12.0	31.3	42
"	17.0	40.0	48				

The data, presented in Table 2, definitely show that addition of ketoglutaric acid to liver slices and homogenates of rats on a low-protein diet, restores to a considerable extent their activity with respect to tyrosine oxidation. If to the liver preparations ketoglutaric and ascorbic acids were both added, then, as a rule, the intensity of tyrosine oxidation reached normal or even exceeded it. In a series of experiments, carried out with liver preparations of normal rats, we were also able to show an increase of tyrosine oxidation by addition of ketoglutaric and ascorbic acids, but this increase usually did not exceed 20-25%. The results of the experiments given above indicate quite convincingly that the primary cause, leading to disturbance of tyrosine oxidation in liver of rats with protein deficiency, is deficiency of ketoglutaric acid. The function of the enzyme system, which catalyzes transamination between tyrosine and ketoglutaric acid in protein deficiency, evidently either is entirely undisturbed, or is disturbed only to an insignificant extent, since addition to liver tissue of the respective animals of ketoglutaric acid alone in many experiments was sufficient to restore completely the process of tyrosine oxidation.

As for the role of ascorbic acid deficiency, as is evident from the data given in Table 2, this is apparent only in the presence of sufficient quantities of ketoglutaric acid. Inasmuch as in the presence of this acid tyrosine transamination by liver preparations of rats with protein deficiency is considerably increased, there are formed relatively large amounts of p-hydroxyphenylpyruvic acid, for whose further conversion to 2,5-dihydroxyphenylpyruvic acid ascorbic acid is necessary. The considerable decrease in content of the latter in liver of rats with protein deficiency must lead under these conditions to accumulation of p-hydroxyphenylpyruvic acid and to inhibition of the process of transamination between tyrosine and ketoglutaric acid, that is, to decrease in intensity of the entire process of tyrosine oxidation.

In a number of experiments we added to liver homogenates of rats with protein deficiency, besides ketoglutaric and ascorbic acids, also glutathione. Addition of this compound, however, increased tyrosine oxidation only to a small extent, from which it can be concluded that the amounts of glutathione, retained in liver of the experimental rats, are yet insufficient to assure a normal course of the process of tyrosine oxidation.

Returning to the question of the role of ketoglutaric acid deficiency as the primary cause of disturbance of tyrosine oxidation in protein deficiency, it is necessary to point out one circumstance, which is of great importance. The fact is, that when we speak of ketoglutaric acid deficiency, this does not at all mean that the amount or concentration of ketoglutaric acid in liver of animals on a low-protein diet is less than in liver of normal animals. On the contrary, determinations of ketoglutaric acid content, specially conducted by N. N. Berezovskaya in our laboratory, showed that in liver of animals with protein deficiency ketoglutaric acid content is even greater than in liver of normal animals (376 and 214 micrograms per 100 g of fresh liver tissue, respectively). This is not surprising, since in the first studies from our laboratory it was shown that oxidation of ketoglutaric acid and other keto acids, which are intermediate products of the citric acid cycle, are appreciably disturbed in animals with protein deficiency. In connection with this in liver tissue a certain amount of ketoglutaric acid can accumulate. If, however, this amount is compared with the amount of free tyrosine which we found in liver (10-15 mg %), then it immediately becomes obvious that the transamination reaction between tyrosine and ketoglutaric acid in liver can proceed normally only if the content of the latter in liver is rapidly restored by means of reactions of the citric acid cycle or other processes.

In the presence of very low ketoglutaric acid content in liver tissue, of primary importance to intensity of the transamination reaction, therefore, is not the change in concentration of ketoglutaric acid but the rate of its formation. In the first studies from our laboratory on the question of the effect of protein deficiency on oxidizing processes in liver, it was shown that many reactions of the citric acid cycle in this deficiency are markedly disturbed [2]. Thus it is possible to assume that the basic factor, causing disturbance of the process of tyrosine oxidation in liver in protein deficiency, is this decrease in amount of ketoglutaric acid, formed in the citric acid cycle. To check this assumption we carried out experiments, in which to liver slices of normal rats and rats with protein deficiency were added not ketoglutaric, but citric acid. The results of these experiments, presented in Table 3, clearly indicate that addition of citric acid to liver slices of normal rats appreciably increases tyrosine oxidation. We have pointed out previously, that addition of ketoglutaric acid to liver slices of normal rats increases tyrosine oxidation by 20-25%, while addition of citric acid produced an increase of 20-50% in intensity of tyrosine oxidation. Thus, addition of citric acid exerted on tyrosine oxidation an even somewhat greater effect than addition of ketoglutaric acid.

TABLE 3

Effect of Citric Acid on Tyrosine Oxidation in Liver Slices.
Weight of slices 250 mg, amount of added citric acid – 4 mg, ascorbic acid – 1 mg

Normal Rats		Rats with protein deficiency	
Tyrosine oxidation in % of initial			
in the absence of citric acid	in the presence of citric acid	in the absence of citric acid	in the presence of citric acid
25	34	8.5	8.5
23	35	11	13
27.5	44	9	9
36	45	12	14
41	47.5	0	0
31	37	5	9
40	46	8	10

A completely different picture was obtained with addition of citric acid to liver slices of rats with protein deficiency. Whereas addition of ketoglutaric acid to liver slices of these animals restored the intensity of tyrosine oxidation to normal in the presence of ascorbic acid, addition of citric acid under the same conditions produced no effect. The results of these latter experiments can be explained only by the fact that in liver of rats with protein deficiency the formation of ketoglutaric acid from citric acid occurs so slowly, that the process of transamination between tyrosine and ketoglutaric acid can not proceed at a normal rate.

The data we have obtained, thus once more emphasize the role of the disturbances of the reactions of the citric acid cycle in the metabolism of individual amino acids. Inasmuch as the transamination reaction with ketoglutaric acid plays a large part also in the metabolism of other amino acids, besides tyrosine, it can be assumed that the mechanism of disturbance of their metabolism in protein deficiency or in other pathological processes, accompanied by decrease in intensity of the citric acid cycle, is analogous to that described above.

SUMMARY

The primary cause, producing disturbance of tyrosine oxidation in liver in protein deficiency, is the decrease in rate of formation of ketoglutaric acid in the citric acid cycle. As a result of this the transamination reaction between tyrosine and ketoglutaric acid is disturbed, which is the first stage in the process of tyrosine oxidation.

Addition of ascorbic acid alone to liver tissue of rats with protein deficiency increases only to a small extent the process of tyrosine oxidation. But addition of ascorbic acid together with ketoglutaric acid completely restores the intensity of tyrosine oxidation.

Decrease in glutathione content in liver is not a limiting factor in the process of tyrosine oxidation in liver in protein-deficient rats.

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SPECTROPHOTOMETRIC METHOD OF DETERMINATION OF SMALL AMOUNTS OF ASCORBIC ACID IN AQUEOUS SOLUTION

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The chemical methods described in the literature for quantitative determination of ascorbic acid (AA) can be subdivided into volumetric and colorimetric.

The basis of the volumetric methods is reduction of an oxidizing agent by means of an equivalent oxidation of AA. Usually AA is oxidized by potassium iodate, iodine or the sodium salt of 2,6-dichlorophenol-indophenol [1]. On the same basis the volumetric methods of so-called ascorbimetry [2] have been developed, which permit determination of trivalent iron, chlorates, iodates, bromates and other oxidizing agents.

The following belong to the group of colorimetric methods: a) the method based on reduction by AA of 2,6-dichlorophenolindophenol and photometric determination of the amount of leuco-compound formed [3], and b) the method based on oxidation of AA by the yellow phosphomolybdic heteropolycomplex and measurement in the visible spectral range of color intensity of solutions of the blue heteropolymolybdic complex formed [4].

The most widely used methods are designed for determination of relatively large amounts of AA. The sensitivity of these methods is inadequate for determination of micro quantitites of vitamin C. It should also be noted that use in analysis of 2,6-dichlorophenolindophenol involves the well-known difficulties and inconveniences, caused by instability of titer and color of the indicator solution and diffuseness of the titration end-point.

On the basis of our studies we have developed a rapid, sensitive spectrophotometric method for determination of very small amounts of AA, based on its oxidation by the yellow phosphomolybdic heteropolycomplex with subsequent determination of the absorption in the near infrared spectral range of the solution of the blue complex formed. A high sensitivity and accuracy of determination of AA can be obtained with observance of optimal conditions for the course of the oxidation-reduction reaction. These conditions should assure more complete reduction of the yellow heteropolycomplex by AA, and, consequently, the obtaining of solutions with the greatest light absorption values. Obviously measurement of this value must be carried out in the spectral range where the molar coefficient of light absorption of the reaction product – the blue phosphomolybdic heteropolycomplex – reaches a maximum, i.e., in the range 825 – 837 mμ. The combination light filter SC-83 which we chose permits isolation of a narrow band of infrared rays of the necessary wave length [5]. Spectral characteristics of the light filter are shown in Figure 1.

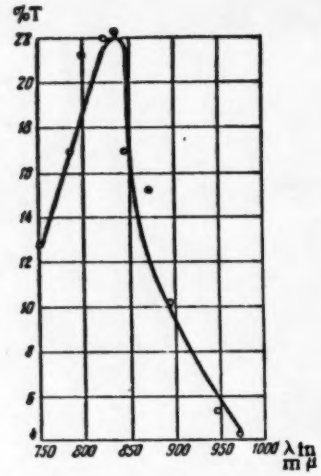

Fig. 1. Transmission curve of combination light filter SC-83 in the near infrared spectral range.

The sensitivity of AA determination by the blue heteropolycomplex depends greatly on pH of the medium, temperature, concentration of reagents and other factors. We have shown that sensitivity and accuracy reach a maximal value with H^+ concentration equal to 1.0 N. With lower concentrations of H^+, AA reduces to molybdenum blue the molybdate not combined in the complex, which is usually present in the experimental solution together with the oxidizer – the yellow heteropolyacid. The presence of molybdenum blue impairs conditions for photometry and reduces accuracy and sensitivity of the determination.

The rate of the process of AA oxidation by the phosphomolybdic heteropolycomplex at room temperature is not great. Heating of the experimental solution in a boiling water bath accelerates the process. Under these conditions the reaction is completed after 10 minutes. Light absorption values of the experimental solutions are stable for 2-3 hours. Stability of the light absorption values of the experimental solutions is significantly increased if the solutions are protected from the action of atmospheric oxygen.

In the reaction of AA with the yellow phosphomolybdic heteropolyacid, the blue phosphomolybdic heteropolycomplex is formed [6]; the stoichiometric relation between the reactants should be 2:1, as is evident from the following equation:

$$2C_6H_8O_6 + H_3[P(Mo_3O_{10})_4] \rightleftharpoons 2C_6H_6O_6 + H_3[P(Mo_3O_9OH)_4]$$

In order to move the reaction equilibrium to the right and assure compliance of the experimental solutions to Beer's law, there must be in the solution a certain excess of oxidizing agent. Experiment has shown that in AA solutions, whose molar concentration is 10^{-7}-10^{-5} mole/liter, the phosphomolybdic complex concentration in these solutions should be approximately 0.65×10^{-3} mole/liter.

DESCRIPTION OF METHOD

Apparatus: 1) Spectrophotometer SF-4 or photocolorimeter of any type, equipped with silver sulfide photoelement of the FESS-U type, with cuvettes of depth 5-6 cm and with combination light filter SC-83. Type FA-52 [7] or FAK [8] photoelectric absorptiometer can also be used; 2) 10-ml graduated glass test tube with ground glass stoppers; 3) chemical glassware (calibrated flasks and burettes, cylinders, etc.); 4) water bath with test tube holders.

Reagents: 1) Phosphomolybdic reagent: 1.03 g ammonium heptamolybdate and 0.047 g K_2HPO_4 are dissolved in 100 ml 1.7 N H_2SO_4 the solution transferred to a 250-ml calibrated flask and filled to the mark with 1.7 N H_2SO_4. It is kept in a bottle of dark glass. 2) Standard AA solution: 10 mg crystalline AA is dissolved in 100 ml distilled water, 10 ml of this solution transferred to a 100-ml calibrated flask and filled to the mark with distilled water. 1 ml of this standard solution contains 10 μg AA.

All reagents must be prepared in water twice distilled over glass, free of traces of metals.

Data for Construction of Calibration Curve

In a series of graduated test tubes, with ground glass stoppers, were placed 6 ml phosphomolybdic reagent and various amounts of standard AA solution, such that the content of the latter in the tubes was 1-30 μg. Volume of liquid in tubes was made up to 10 ml with twice-distilled water. In one of the tubes, to serve as control ("zero") solution, no AA was added. Tubes were stoppered, shaken and placed without stoppers for 10 minutes in a boiling water bath. Then they were cooled under a stream of tap water, the volume made up to 10 ml (with twice-distilled water) where necessary, again shaken and subjected to photometry. For this, one of a pair of previously calibrated cuvettes of the photoelectric potentiometer was filled with control solution, placed in position, and the galvanometer indicator set at zero or on the maximal scale division, depending on construction of the

instrument and method of measurement. Constancy of the instrument reading for the control solution (I_0) must be checked before each absorption measurement of the experimental AA solution. At the same time the second cuvette of the pair was filled with the first standard solution and after measurement of the I_0 value, I_1 for this solution was determined. In the same way instrument readings (I_2, I_3...I_n) were made for the other standard AA solutions.

The calculated light absorption values ($E = \log \frac{I_0}{I_{1,2,3,\ldots n}}$) and the corresponding exact AA concentrations in the standard solutions were used for calculation of the calibration curve (abscissae – AA concentration in μg; ordinates – light absorption value – E).

A typical calibration curve for an aqueous AA solution is given in Figure 2 (line A). In the same figure is given the calibration curve for AA, dissolved in 10% NaCl solution (line B). Depth of the liquid layer in the cuvette was 6 cm, cuvette volume – 5 ml.

The straight-line character of the calibration curve indicates compliance to Beer's law of the experimental solutions within AA concentration limits 1-30 μg in 10 ml of solution. Sensitivity of the determination – 0.5 μg AA.

ANALYSIS PROCEDURE

For determination of AA concentration in a solution 6 ml of phosphomolybdic reagent was placed in each of two graduated test tubes, then in one of them 1–4 ml of the experimental solution (depending on the estimated AA concentration) and liquid volume in the tube made up to 10 ml with twice-distilled water. To the other control tube distilled water was added instead of AA. Tubes were stoppered, shaken and placed for 10 minutes in a boiling water bath. All further procedures for preparation of solutions for photometry and measurements were carried out as has been described above.

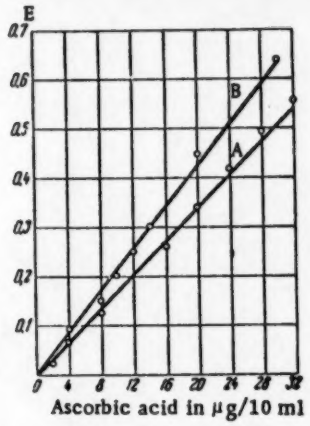

Fig. 2. Calibration curves for ascorbic acid solutions:
A) in water; B) in 10% NaCl.

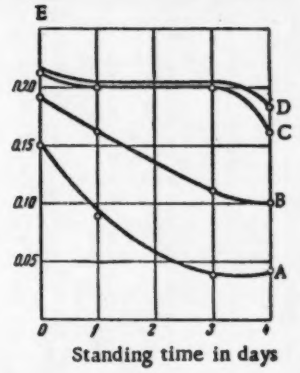

Fig. 3. Relation of absorption values of ascorbic acid solutions to NaCl concentration and standing time of solution:
A) Ascorbic acid solution in water;
B, C, and D) ascorbic acid solutions in 1, 5, and 10% NaCl.

From the instrument readings and the calibration curve, the AA content was determined in the experimental solution. Total analysis time was 18-20 minutes. In serial analysis the time for one determination can be significantly shortened. Certain data, which we obtained in checking the accuracy of the method, are given in the table.

Spectrophotometric Determination of Ascorbic Acid (AA)

AA in μg		Difference in μg
added	found	
1	0.5	−0.5
2	1.8	−0.2
5	5.4	+0.4
10	9.8	−0.2
15	15.2	+0.2
25	25.4	+0.4

Using this method, we made a study of the stability of dilute AA solutions in distilled water and in 1, 5 and 10% NaCl. Concentration of AA in each of these solutions was 5 mg/liter.

For analysis 1 ml of each experimental solution was taken. The final volume of the sample was made up with water to 5 ml. The solutions were studied at the time of preparation and after one, three and four days. Results of the measurements are given graphically in Figure 3.

As is evident from the data presented, stability of dilute AA solutions increases in proportion to increase in NaCl concentration up to 10%. Obviously for this reason the calibration curve for AA in 10% NaCl is located higher than the corresponding curve for the aqueous AA solution (see Figure 2).

In conclusion it should be noted that for oxidation of AA, as well as the yellow heteropolyacid, another reagent can be used – an acid solution of ammonium heptamolybdate. The latter is reduced by AA and other reagents to molybdenum blue, with maximal light absorption in the range 700-725 mμ [5]. Optimal concentration of H^+ in AA oxidation by a 2×10^{-3} M ammonium heptamolybdate solution is 0.1N. Photometry should be carried out in the range 700-725 mμ.

SUMMARY

A spectrophotometric method is proposed for determination of small amounts of ascorbic acid in aqueous solution. The method permits determination of microgram quantities of ascorbic acid with an accuracy satisfactory for such concentrations.

The method is based on oxidation of ascorbic acid by the yellow phosphomolybdic heteropolycomplex and measurement of the light absorption value of the reaction product – the blue phosphomolybdic heteropolycomplex solution – in the near infrared spectral range (λ_{max} = 825-837 mμ).

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USE OF IONOPHORESIS IN COMBINATION WITH PAPER CHROMATOGRAPHY FOR IDENTIFICATION OF AMINO ACIDS IN NATIVE PROTEIN HYDROLYZATES

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For study of the amino acid composition of proteins in the last ten years a constantly wider use has been made of physicochemical methods of separation of amino acid mixtures: ionophoresis, adsorption and distribution chromatography, which is explained by the relative simplicity and accuracy of these methods.

Distribution chromatography of amino acids on paper is the most effective of presently existing methods for qualitative and quantitative analysis of the amino acid composition of proteins. But this method does not always lead to complete separation of all amino acids in the mixture to be analyzed in one experiment. For complete separation of complex mixtures a number of experiments must be carried out with use of various solvents, which makes the procedure lengthy and laborious. Especially difficult to separate in this case are the diamino acids and such monoamino acids as leucine, isoleucine, phenylalanine.

To remove this disadvantage the method of distribution chromatography on paper is sometimes combined with other methods of separation. Most frequently resorted to is a combination of distribution chromatography and ionophoresis of amino acids, in which ionophoresis either precedes distribution chromatography, or is combined in one operation with chromatography.

Thus, Solovyev [1] first separated an amino acid mixture by ionophoresis into groups of neutral, acid and basic products. Each group was then analyzed by the method of distribution chromatography on paper. Haugaard and Kroner [2] combined ionophoresis and chromatography in one operation.

Behavior of amino acids during paper ionophoresis at various pH values was studied in detail in work by Durrum [3].

In analyzing an amino acid mixture, obtained upon hydrolysis of lipoproteins of human brain tissue, we encountered the fact of insufficiently clear separation of amino acids by the method of distribution chromatography on paper. Obviously the admixture of various substances in hydrolyzate of brain lipoproteins interferes with separation of amino acids by this method. Attempts to get rid of interfering substances by extraction of the amino acids from dry residue of the hydrolyzate with acid acetone were not successful.

In this connection the separation of this mixture was carried out by a combination of two methods – ionophoresis and distribution chromatography on paper.

EXPERIMENTAL

At the beginning of the work we tried to conduct ionophoresis and distribution chromatography in one operation.

Strips of filter paper 570 × 120 mm were saturated in M/15 phosphate buffer, pH 6.2. Excess liquid was removed from the paper strips by pressing them between several sheets of filter paper. Along the long edge of the strip, electrodes were attached which consisted of aluminum strips 7 mm wide and 0.025 mm thick. The electrodes occupied $^3/_4$ the length of the paper strip. The moist paper strip with electrodes was placed in

solvent (in a chromatographic vessel). Phenol, saturated with water, served as solvent, voltage 100 v, current strength 18-20 milliamperes.

However, there was not sufficiently clear separation of amino acids by this method; first, phenol flow through the paper, saturated with buffer salts, was very slow; second, the salts, present in the paper as a result of its saturation with phosphate buffer, strongly interfered with separation of amino acids in phenol. As a consequence of this the ionophoresis procedure was separated from the chromatography. First the separation of amino acids was tested by paper ionophoresis in buffer solutions of various pH values.

In the center of a strip of chromatographic paper 250 x 40 mm was applied the experimental amino acid or amino acid mixture, dissolved in water. The solvent was allowed to dry and then the strip was placed in an electrophoretic vessel for separation of proteins on paper. When the strips were saturated with buffer, ionophoretic separation was conducted with voltage 350–400 v and current strength 0.5–1 milliampere per cm width of paper, for 6 hours.

After completion of separation the paper strips were dried in a drying cabinet at 80–90°. Location of amino acids was determined by spraying strips with 0.1% ninhydrin solution in butyl alcohol containing acetic acid. Of the buffer solutions with various pH values tested, we chose veronal buffer, pH 8.62, since results of separation in this buffer were the most satisfactory.

In Figures 1 and 2 are given the results of study of the behavior of standard amino acids under our experimental conditions.

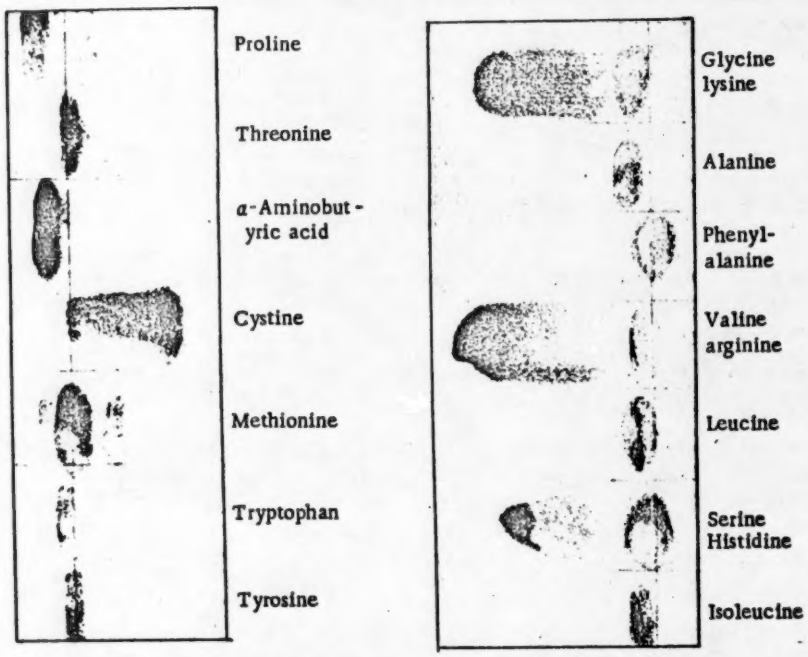

Fig. 1. Distribution of amino acids on paper after separation in veronal buffer, pH 8.62. Duration of separation – 6 hours.

As is evident from the figures, first of all there occurs a clear separation of amino acids into three groups. Basic amino acids moved toward the cathode a considerable distance from the mid-line. In addition 6-hour ionophoresis led to individual separation of the basic amino acids. Arginine was closest to the cathode, lysine followed farther behind and histidine moved considerably slower. The dicarboxylic acids moved toward the anode; aspartic acid outdistanced glutamic acid. Also considerably displaced from the mid-line toward the

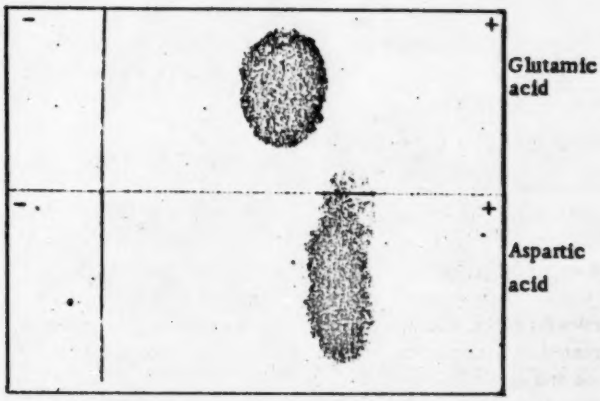

Fig. 2. Distribution of aspartic and glutamic acids after 6-hour separation in veronal buffer, pH 8.62.

anode was cystine, but still lagging far behind the dicarboxylic acids. The amino acids threonine, methionine, tyrosine, serine and phenylalanine remained in their original position even upon longer ionophoresis. Proline, α-aminobutyric acid, tryptophan, glycine, alanine, valine, leucine and isoleucine, after 6-hour separation, were insignificantly displaced from the mid-line toward the cathode.

While studying the behavior of indiyidual amino acids during paper ionophoresis we attempted to separate a complex mixture of amino acids, in particular a protein hydrolyzate. In this we obtained a clear separation of amino acids into three groups – neutral, basic and dicarboxylic acids. In the group of basic amino acids there was attained, in addition, satisfactory separation into three individual amino acids. The dicarboxylic acids also gave two zones, corresponding to glutamic and aspartic acids. The zone corresponding to cystine was very prominent. The monoamino acids were distributed over a wide zone, occupying a central position, and in part displaced toward the cathode.

The advantage of such separation of amino acids is that the presence of salts in the mixture to be separated does not interfere with analysis, while in separation of amino acids by the method of distribution chromatography salts are a great hindrance. For instance, we succeeded in separating amino acids of urine without preliminary removal of salts. This method can be used for a very simple and very rapid quantitative determination of the three groups of amino acids in proteins and in other experimental material.

Having obtained entirely satisfactory results in ionophoretic separation of amino acids, we tried to combine ionophoresis and the method of distribution chromatography on paper. Chromatographic separation of amino acids in our procedure preceded ionophoresis.

In final form the combination of the two methods consisted in the following. On a sheet of chromatographic paper 40 x 30 cm, 5 cm from the upper edge of the sheet along the mid-line, were cut out two rectangular openings 20×5 mm, 5 mm apart. On the narrow piece separating the openings was applied the solution of experimental amino acid mixture, then separation was carried out by the method of distribution chromatography. In this work both descending and ascending methods were used. Water-saturated phenol or butyl alcohol – acetic acid mixture (4:1:5) served as solvent. Two chromatograms were always set up, one of which was developed immediately after chromatographic separation, which made it possible to cut off those parts of the paper where there were no amino acids, and thus to decrease the size of the chromatogram, which was to be subjected to further separation.

The chromatogram freed of solvent was placed in an electrophoretic vessel, where in addition ionophoretic separation of amino acids was conducted for 6 hours. Then after drying, the chromatogram was developed with ninhydrin solution.

As a result a two-dimensional chromatogram was obtained with sufficiently clear separation of basic and dicarboxylic acids. We have used the method described above for identification of the amino acids of lipoproteins of human brain tissue.

Lipoproteins were obtained according to the method proposed by one of us for their extraction from bacterial cells [4].

The brain of an individual, who died from trauma, was washed free of blood, brain membranes and large vessels were removed and the brain tissue was carefully divided into white and gray matter. For experiment were taken 80 g of white and 20 g of gray matter. Then both materials were ground with twice their weight of freshly prepared Na_2SO_4. The mass was spread in a thin layer on glass and after thorough drying was again ground and carefully extracted with ether, and then twice with chloroform. The residual mass after removal of traces of chloroform was extracted with a mixture of ethyl alcohol and chloroform (1:1). The alcohol-chloroform extract was filtered and concentrated in wide crystallizing dishes in air at 18°. The residue after evaporation of solvent was washed with ether.

Thus preparations were obtained of the lipoproteins of gray and white matter of brain. Preparations were of yellow color and friable consistency. Neither preparation was completely soluble in the original solvent or other organic solvents. A portion, soluble in alcohol-chloroform mixture, gave weak biuret and positive xanthoproteic reactions. The hydrolyzate, obtained by boiling with 6N H_2SO_4 for 24 hours, gave positive ninhydrin, Sakaguchi, xanthoproteic reactions.

For removal of the lipoid component from the lipoproteins both preparations were heated with a saturated solution of picric acid in a boiling water bath for one hour. Acetone was then added and the precipitates thus formed were removed by centrifugation. To remove excess picric acid they were washed with ether and then acetone.

By this treatment there were separated from the lipoprotein of white matter of brain solid fatty acids of slightly yellowish color, from the lipoprotein of gray matter – liquid fatty acids of dark brown color. The substance insoluble in acetone represented chiefly the protein portion of the lipoproteins, whose amino acid composition we studied.

The protein portion of the lipoproteins of gray and white matter of brain was hydrolyzed with 6N HCl for 24 hours. The hydrolyzates were diluted with water, filtered and evaporated on a boiling water bath to as complete as possible removal of hydrochloric acid.

With the purpose of comparing the results obtained the separation of the amino acids of the hydrolyzate was carried out by the method of one-dimensional chromatography, two-dimensional chromatography, the method of ionophoresis and a combination of chromatography and ionophoresis. Concentrations of the experimental material were in all cases the same.

One-dimensional chromatography permitted detection of the presence of the following amino acids: cystine, aspartic acid, threonine, glutamic acid, alanine, tyrosine, valine and phenylalanine. A spot was observed on the chromatogram corresponding to the basic amino acids, but it was impossible to conclude that all three basic amino acids were present. These amino acids were found both in the hydrolyzate of lipoprotein of gray matter of brain, and of lipoprotein of white matter. Also insufficiently clear was the separation of aspartic acid, threonine and glutamic acid – the spots, corresponding to these amino acids, ran together.

The two-dimensional chromatogram (solvents phenol – butyl alcohol-acetic acid) gave less satisfactory results than one-dimensional chromatography. Even considerably higher hydrolyzate concentrations did not lead to appearance of a greater number of spots. The two-dimensional chromatogram of hydrolyzates of lipoproteins of gray and white matter had in all four spots. Three spots represented serine, glycine and basic amino acids. The fourth spot we did not identify. It was characterized by a position in front of serine with movement in butyl alcohol-acetic acid, and in phenol it was located at the same level as serine.

Ionophoresis of the experimental hydrolyzate permitted detection of all three basic amino acids: arginine, lysine and histidine. Clearer separation of glutamic and aspartic acids was also obtained.

In Figure 3 are presented ionophoregrams of hydrolyzates of lipoproteins of gray and white matter.

Fig. 3. Separation by ionophoresis of amino acids of hydrolyzate of lipoproteins of white (A) and gray (B) matter of brain.

Finally, the combination of the methods of chromatography and ionophoresis made it possible for us in one experiment to obtain satisfactory separation of an amino acid mixture. An unknown amino acid, detected on the two-dimensional chromatogram, was not identified.

As a result of the study it has been shown that the amino acid composition of lipoproteins of gray and white matter of brain is the same. In them were detected cystine, arginine, lysine, histidine, aspartic acid, threonine, serine, glycine, glutamic acid, alanine, tyrosine, valine, phenylalanine and one unidentified amino acid.

Judging by color intensity of spots, corresponding to the various amino acids, certain conclusions can be made concerning the quantitative content of individual amino acids. Thus, in both hydrolyzates the concentration of basic amino acids exceeds the amount of dicarboxylic acids. The amount of basic and dicarboxylic acids in the hydrolyzate of lipoprotein of gray matter is greater than in the hydrolyzate of white matter. In the latter the amount of neutral amino acids was relatively greater.

SUMMARY

The behavior was studied of various amino acids upon separation by the method of paper ionophoresis. By this method a complex mixture of amino acids can be separated into a number of groups. Such separation is distinguished by simplicity and rapidity of execution.

A method has been developed for amino acid separation, which consists in preliminary separation by the method of one-dimensional chromatography with subsequent separation by the method of ionophoresis. Such combination of the two methods is especially effective for separation of basic and dicarboxylic acids.

By means of the method developed the amino acid composition was studied of lipoproteins of gray and white matter of brain. There were detected in these preparations cystine, arginine, lysine, histidine, aspartic acid, threonine, serine, glycine, glutamic acid, alanine, tyrosine, valine, and phenylalanine. One amino acid present in both hydrolyzates was not identified.

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ALLOXAN DESTRUCTION IN BLOOD OF ANIMALS

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It has been established by many studies [1] that alloxan is an excellent means for production of experimental islet diabetes. By utilizing this type of diabetes, it has been possible to clarify many unsettled questions associated with the origin and treatment of diabetes. A thorough study of the mechanism of action of alloxan is of great theoretical interest and may have a certain practical importance. The possibility of alloxan formation in man is not excluded. In the blood of normal man traces of alloxan can be found [1].

Alloxan introduced into the organism is very rapidly destroyed. Lazaris and Ugodchikova [2] have established that after subcutaneous injection of alloxan into rats, it is not possible to detect it in the blood. Experiments with parenteral injection of alloxan into rats also provide evidence of its rapid destruction. However, Lazaris and Ugodchikova, in studying alloxan conversions in blood, could not completely solve this question, since they did not have available a sufficiently sensitive and specific method by which to detect minimal amounts of alloxan.

In order to study the rate of alloxan destruction in blood we used the polarographic method proposed by us for its determination in whole blood. This method makes it possible to follow alloxan decomposition from the moment of its introduction into the blood and does not require preliminary precipitation of blood proteins. The latter is very important, since alloxan is rapidly destroyed – 3-4 minutes after its introduction into the blood its quantitative determination is impossible.

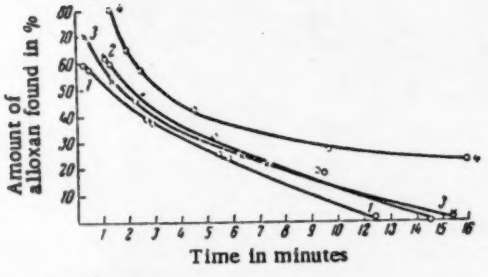
Alloxan in whole blood was determined with background of buffer solution within limits of pH 6.2-5.5, at 16°. The half-wave potential of alloxan under these conditions is equal to 0.1 V. The polarographic waves of alloxan and oxygen nearly coincide, hence oxygen interferes with the determination. To eliminate oxygen, electrolytic hydrogen was passed through the solution. It has been established [3] that whole blood in the amount of 0.2 ml per 2 ml of background solution after further passage of hydrogen does not change the background wave. The diffusion current was always determined with account taken of the background wave.

The method of polarographic determination of alloxan which we have used has made it possible to study its rate of destruction in blood in vitro and in vivo with time. In the in vitro experiments blood of white rats was used, stabilized with sodium fluoride. A freshly prepared, approximately 0.1 M solution of alloxan in physiological solution was used in the experiments. Blood taken after decapitation of animals in the amount of 0.3 ml was placed in a small crucible, covered with a watch glass and kept for 5 minutes in a water bath at 37°. Then 0.1 ml of alloxan solution was added to the blood which was left in the water bath for a definite time. Then 0.1 ml of blood was removed with a micropipet and placed in the background solution, through which hydrogen had first been passed. The time from the moment of introduction of alloxan into the blood until introduction of blood into the background solution was timed with a stopwatch. Hydrogen was again passed through the background solution and blood for 10 minutes, after which the polarogram was taken with galvanometer sensitivity S = 1/5, capillary characteristic m = 1.889 mg/sec and t = 3.6 seconds.

Results of the experiments, dealing with study of the rate of alloxan destruction in whole blood in vitro, are given in Table 1 and in the figure (Curve 1). It can be stated that the greater part of the alloxan is destroyed within 5-6 minutes after introduction. In 9-10 minutes only traces of alloxan, and in 11 minutes 30 seconds no alloxan can be found in the blood.

In all the tables and in the figure the amount of alloxan determined by the polarographic method is given in percent of the amount of alloxan added to 1 ml of blood (serum, plasma, buffer). In order to obtain the absolute amount of alloxan, which was determined in each separate case, it must be taken into account that in this method 0.1 ml of the blood to be analyzed was added to the background solution.

Alloxan solutions are stable at pH below 3.5. With increase of pH of the solution the stability is markedly reduced [4]. It was of interest to determine whether alloxan decomposes only under the influence of the blood pH or whether other factors influence its decomposition.



Curves of Alloxan Destruction:
1) in whole blood; 2) in blood plasma; 3) in blood serum; 4) in phosphate buffer solution.

To answer this question, in addition to alloxan decomposition in whole blood we also studied the rate of its decomposition in plasma, serum, and phosphate buffer with pH that of blood. Experimental conditions and quantitative relations were exactly the same as in the experiment with whole blood in vitro. Results of our observations given in the figure indicate that alloxan destruction occurs most rapidly in whole blood, then in plasma and blood serum (Curves 2 and 3), where the rate of destruction is the same, and, finally, most slowly in buffer solution (Curve 4).

It can be assumed that the more rapid destruction of alloxan in whole blood, in comparison with plasma, is associated with the presence in blood of erythrocytes. It is known that erythrocytes contain relatively large quantities of glutathione, which reacts very actively with alloxan. This is the probable explanation of the more rapid destruction of alloxan in whole blood.

TABLE 1

Rate of Alloxan Destruction in Whole Blood in Vitro.

Time after addition of alloxan to blood	Alloxan added in mg/ml of blood	Alloxan found in %	Alloxan added in mg/ml of blood	Alloxan found in %	Time after addition of alloxan to blood	Alloxan added in mg/ml of blood	Alloxan found in %	Alloxan added in mg/ml of blood	Alloxan found in %
0'45"	8.9	59.0	5.7	59.6	4'00"	8.9	33.0	—	—
0 54	—	—	5.7	58.0	5 30	—	—	5.7	26.2
0 55	8.9	56.0	—	—	5 50	8.9	23.1	5.7	24.6
1 10	8.9	53.8	—	—	9 12	8.9	Traces	5.7	Traces
1 35	8.9	52.0	—	—	9 40	—	—	5.7	Traces
2 45	—	—	5.7	38.6	11 30	8.9	0	5.7	0
2 55	—	—	5.7	37.0					

For further study of the mechanism of action of alloxan, of particular interest are the dynamics of its destruction in vivo. To answer this question we investigated alloxan transformation in blood in vivo in experiments on rats and guinea pigs.

Animals were opened under ether narcosis and alloxan in physiological solution was injected into the inferior vena cava or portal vein (the amount of alloxan injected was calculated in mg per 1 ml of blood of the animal). Blood was taken from the heart after various time intervals and its alloxan content determined. Conditions of determination were the same as in previous experiments.

TABLE 2

Rate of Alloxan Destruction in Blood of White Rats in Vivo
Alloxan was injected into inferior vena cava.

Weight of rats in g	Time after introduction of alloxan	Alloxan given in mg/ml of blood	Alloxan found in %	Weight of rats in g	Time after introduction of alloxan	Alloxan given, mg per ml blood	Alloxan found in %
282	1'30"	4.60	47,0	176	3'14"	7.40	Traces
242	1 33	5.40	31.2	102	3 25	6.42	Traces
182	1 38	7.15	35.0	235	5 15	2.80	0
242	2 06	5.32	25.6	100	8 00	6.42	0
163	3 00	7.75	19.4	178	9 00	3.65	0

TABLE 3

Rate of Alloxan Destruction in Blood of White Rats in Vivo
Alloxan was injected into portal vein.

Weight of rats in g	Time after introduction of alloxan	Alloxan given in mg/ml of blood	Alloxan found in %	Weight of rats in g	Time after introduction of alloxan	Alloxan given in mg/ml of blood	Alloxan found in %
285	1'20"	4.60	31,8	235	2'03"	5.56	21.2
170	1 35	7.70	46,5	255	3 40	5.10	Traces
130	1 37	5,00	38.7	117	5 54	5.50	0
132	1 55	5,00	23,6	120	6 00	5.50	0

TABLE 4

Rate of Alloxan Destruction in Blood of Guinea Pigs in Vivo
Alloxan was injected into inferior vena cava.

Weight of rats in g	Time after introduction of alloxan	Alloxan given in mg/ml of blood	Alloxan found in %
335	1'45"	6.90	38.2
535	3 05	6.40	32.8
265	4 45	7.10	Traces
320	5 20	6.40	0

In Table 2 are given the results of experiments carried out on 10 white rats, given alloxan via the inferior vena cava. From the table it is evident that within 3-5 minutes after introduction of alloxan, it can not be determined quantitatively in the blood. With injection into the portal vein (Table 3) within 3 minutes 40 seconds it likewise can not be quantitatively determined. In the last series of experiments, carried out on four guinea pigs, the rate of alloxan destruction was studied after injection into the inferior vena cava (Table 4). In these experiments after 4 minutes 45 seconds only traces of alloxan were found.

All these experiments indicate the rapid alloxan destruction in the animal. However, its presence in the blood for 3 minutes is sufficient for it to exert its diabetogenic action. Summarizing the results of all our experiments, it can be considered established that alloxan, injected into the blood of animals, is destroyed considerably more rapidly than in vitro.

SUMMARY

The greater part of the alloxan, added to blood of white rats in vitro, is destroyed within 5-6 minutes after introduction. The destructive capacity of blood in vitro is chiefly due to its slightly alkaline reaction. However, other factors also, which require special study, are of certain significance. Destruction of alloxan, injected directly into the blood of animals in vivo, occurs considerably more rapidly than in blood in vitro, – the greater part of the alloxan is destroyed under these conditions within 3 minutes.

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THE MECHANISM OF PROTEIN DENATURATION

PROPERTIES OF A GLOBULAR PROTEIN STABILIZED BY A DENATURING FACTOR

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In previous papers [1] it was shown that the denaturation process can stop at certain levels in a false equilibrium. This occurs because of the fact that under the influence of a denaturing agent (urea) part of the native protein in the system is stabilized, i.e., is modified in such a way that its resistance is considerably increased. This phenomenon has been designated as "denaturation stabilization" [1]. In the present paper the properties of the stable form of protein are described, obtained by means of denaturation stabilization – by the action of a denaturing agent.

False equilibria and denaturation stabilization were shown previously for three proteins – egg and serum albumins and edestin [1]. The experiments in the present paper were carried out only on egg albumin, which has in this case a number of advantages: the properties of this protein have been thoroughly studied, it is irreversibly denatured, does not dissociate in urea solutions, and changes occurring under the influence of stabilizing treatment are very stable.

The stabilized form of egg albumin was obtained by the action of urea, that is, as usual, denaturation was carried out in the system, egg albumin – urea, up to establishment of false equilibrium. Then the mixture was allowed to stand for various intervals of time, whereupon the stabilized part of the protein was isolated from the system. This treatment was carried out at room temperature, 5.0-9.0% albumin solutions were used, usually of pH~7.0; 300-500 mg urea (dry, chemically pure) was added per 1 ml of solution.

Method of Isolation of Stabilized Protein

For investigation of the properties of modified egg albumin it was necessary to develop a method for its isolation from the equilibrium systems. Isolation was performed by various means although based on a common principle, the denatured and native (stabilized) parts of the protein being separated by means of isoelectric precipitation.

After testing a number of methods (described in detail previously [1]), we adopted the following procedure: 50 ml of equilibrium mixture of protein – urea was placed in a cellophane sac and most of the urea was removed by dialysis against distilled water for 24 hours. Then there was introduced directly into the sac 2.0 ml of a mixture, consisting of 1.5 ml of acetate buffer, pH 4.7, and 0.5 ml of saturated $(NH_4)_2SO_4$ solution. Thereupon, the denatured part of the protein immediately precipitated as a coagulate. Dialysis was continued until ammonia ions were removed, then the coagulate was removed by centrifugation, the solution yielding the undenatured "fraction," containing the stabilized egg albumin.

From such material we obtained the first data on the character of the stabilized protein, established its high resistance to denaturing agents, high optical rotation, increased viscosity, etc. These data were of a preliminary character, but made it possible to obtain much accurate information.

Then a series of observations turned our attention to the fact that in addition to stabilized albumin, there were in the isolated "fraction" protein decomposition products, formed also, obviously, as a result of the action of urea (the experiments were described in detail previously [1]). The amount of decomposition products was

quite considerable and this compelled us to develop another, better method of isolation of the stabilized protein, in which it could be obtained without admixture of decomposition products. This proved possible by salting out the protein from the undenatured fraction with ammonium sulfate.

After a number of experiments the following method was developed. To 50 ml of protein – urea mixture, which had attained a false equilibrium, three volumes of isoelectric precipitant for egg albumin was added – 0.1 M acetate buffer, pH 4.7 containing 10% $(NH_4)_2SO_4$. After the mixture had stood one hour at room temperature, the precipitated denatured protein was removed by centrifugation and to the clear filtrate was added five volumes of saturated ammonium sulfate solution. The degree of salt saturation was about 0.83. Preliminary experiments showed that at this salt concentration all the stabilized protein was completely isolated. After addition of the salting-out solution the mixture with precipitate of stabilized protein was left overnight at room temperature. Then the precipitate was removed by filtration (centrifugation proceeds with great difficulty!), taken from the filter with a porcelain spatula and transferred to a glass beaker, to which was added a minimal amount of water, only enough so that the slurry formed after mixing could be taken up in a pipette with a wide terminal opening. The fluid slurry was transferred to a cellophane sac and dialyzed, usually for several days, against twice-distilled water. Several hours after removal of most of the ammonium sulfate the solution in the sac became clear. Completion of dialysis was determined by disappearance of ammonia ions. Various properties of the solution of stabilized protein (2.8-4.0%) obtained were investigated.

Resistance of Stabilized Form of Egg Albumin to Diverse Denaturing Agents

Of great interest is the question whether the action of any one denaturing agent (e.g., urea) stabilizes proteins to other, essentially different denaturing factors (e.g., alcohol, solubilizing salts such as KCNS, etc.). In other words, does the presumed new macrostructure formed by gradual rearrangement possess a general resistance to diverse factors or is it resistant only to that factor under whose influence it was formed? To answer this question the stable modification of egg albumin that was obtained by the action of urea and its denaturation by alcohol, sodium salicylate, potassium thiocyanate, and urea at increased concentration was studied. Simultaneously under the same conditions, the denaturation was observed of freshly crystallized egg albumin, analogous to the initial preparation from which the modification was obtained.

Experiments were carried out in the following way. The dialyzed solution of modified egg albumin contained 1.5-2% protein; a solution of the ordinary crystalline egg albumin of the same concentration was prepared, and used for the control experiments. Both solutions (experimental and control) were poured into four test tubes, 5 ml in each, and denaturing agents were added: sodium salicylate – 100 mg per 1.0 ml of solution, alcohol 2.5 ml per 5.0 ml of solution, potassium thiocyanate 2.5 ml of a 50% solution per 5.0 ml of solution, and urea 600 mg per 1.0 ml (whereas stabilization was carried out at a lower concentration, 40 mg per 1.0 ml of protein solution). Denaturation was conducted at room temperature (20 ± 1°). In the experiment with alcohol the protein solution and alcohol were cooled to 5° in order to decrease the rate of the rapidly occurring process and to observe it more easily at the beginning. The course of denaturation in all eight samples was followed by determining the amount of denatured protein in the system by means of isoelectric precipitation [2]. The amount of protein precipitated was estimated by the biuret reaction. The data obtained (typical experiment) are presented in Figure 1. The experiments were repeated with various protein preparations and gave results in complete agreement.

As is seen from the curves in Figure 1, the modified form of protein is distinguished by high resistance to any of the denaturing agents used, and not only to the one under whose action it was obtained. This could be seen even in the external appearance of the experimental mixtures. Thus, in the experiment with salicylate, in the sample containing control protein, there was formed a dense turbidity, while in the solution of stabilized protein addition of salicylate produced no changes. The same thing, although to less degree, was observed in the solutions with alcohol and with KCNS.

Resistance to such diverse factors as sodium salicylate, alcohol and potassium thiocynate, which denature protein in completely different ways (e.g., breaking hydrogen bonds or acting on bonds between nonpolar radicals),

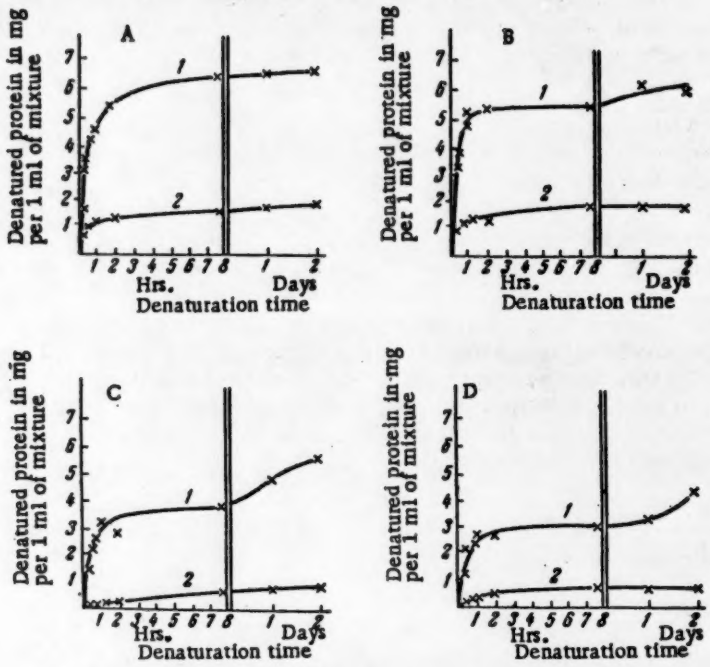

Fig. 1. Denaturation of stabilized form of egg albumin (obtained by treatment of protein with urea) – by alcohol, sodium salicylate, potassium thiocyanate and urea at increased concentration:
1) denaturation of crystalline egg albumin (control); 2) denaturation of stabilized form; A) urea; B) alcohol; C) sodium salicylate; D) potassium thiocyanate.

gives evidence that the stabilization of molecules which has occurred is a general change of the whole macrostructure, associated, as we have suggested, with change in the network of bonds, forming the characteristic arrangement of polypeptide chains.

Data on Solubility ("Ability to be Salted Out ") of Stabilized Form of Protein

In the following series of experiments in order to characterize the changes of the stabilized form of egg albumin in comparison with the unchanged crystalline protein, the degree of solubility in salt solutions of varying concentration was studied. Solubility of proteins, as is their "ability to be salted out" of solution, is a sensitive indicator of their properties, in particular of structural differences, the associated changes of hydrophilia, etc. We carried out fractional salting out of preparations of modified and initial protein with $(NH_4)_2SO_4$, isolating and determining quantitatively the portion of protein insoluble in the concentration ranges 0-33, 33-50, 50-60, 60-67, 67-77% saturation of the salt solution.

To ~30% protein solution pH 4.9-5.1, was added a saturated solution of ammonium sulfate in the amount necessary to obtain the degree of saturation equal to 33%. In the crystalline egg albumin no visible changes occurred, but in the solution of the modified form there was a small precipitate. The latter was removed by centrifugation. The clear liquid was poured from the precipitate, another quantity of ammonium sulfate was added, such that the degree of saturation was 50%. Simultaneously a control solution of crystalline egg albumin was treated in the same way. After the second addition of saturated $(NH_4)_2SO_4$ in the ordinary egg albumin again no changes were observed, while in the stabilized protein a new precipitate formed, which was removed in the same way as the first. Only the increase of degree of saturation of the solutions to 60% led to salting out of part of the protein in the control. In this range of salt concentration (i.e., 50-60% saturation) most of the modified

protein usually precipitates also. Precipitates were removed in the usual way and the experiment was concluded, after collecting the protein fractions, salted out at 60-67% and 67-77% saturation. In the latter concentration range practically nothing precipitated from the control solution, but from the experimental – a small, but significant amount of protein. All precipitates were washed with ammonium sulfate solutions of the corresponding concentration, again separated by centrifugation, and the amount of protein in each was determined by the biuret reaction. The data obtained were expressed in percent of the total amount of protein contained in the initial solution. Several typical experiments are shown in Table 1.

TABLE 1

Salting Out with Ammonium Sulfate of Protein from Solutions of Crystallized Egg Albumin and of Its Modified Form, Obtained as a Result of Denaturation Stabilization
(in % of amount dissolved)

No. of experiment	Preparation	Degree of saturation of solution with ammonium sulfate in %				
		0-33	33-50	50-60	60-67	67-77
1	Crystalline egg albumin (preparation 1)	0	0	32.8	63.9	3.3
2	Crystalline egg albumin (preparation 2)	0	0	13.8	83.8	2.4
3	Stabilized form (preparation 1)	3.8	13.0	45.4	31.3	6.5
4	Stabilized form (preparation 2)	8.0	13.7	60.0	13.2	6.1

We see that the "ability to be salted out" of the modified (stabilized) form of egg albumin has changed considerably in comparison with the ordinary, initial protein. The data in Table 1 show that as a result of stabilization there has appeared an easily salted out portion of protein, corresponding with respect to this property, to globulins, which precipitate from solution with ammonium sulfate saturated to 50%. In the initial protein preparation there is no such component. Together with this in the modified preparation there is an increase of the protein fraction of high solubility, the most difficult to salt out (in the range 67-77% saturation). The total solubility of the modified protein is decreased in comparison with the initial. More than 20% of the material precipitates out with salt saturation lower than 50%; there is considerable increase on the part of the preparation salted out in the range 50-60% saturation. As is evident from the control experiments (1, 2, Table 1), ordinary egg albumin is distinguished by much greater homogeneity. Practically all the protein precipitates in the range 50-67% saturation, which entirely corresponds to the conditions under which it was obtained [3]. Here the greater part of the protein preparation precipitates at 60-67% $(NH_4)_2SO_4$ saturation, while for the stabilized form the "ability to be salted out" is displaced in the direction of lower salt concentrations and the greater part of the protein precipitates at 50-60% saturation of the solution.

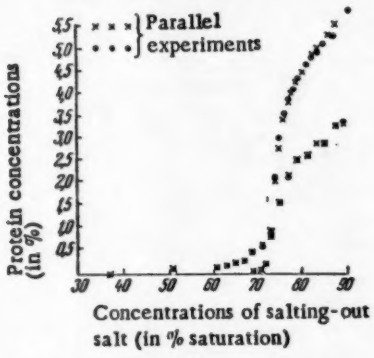

Fig. 2. Salting-out curves of modified and initial (crystalline) egg albumin.

Apparently the most important observation in these experiments is the fact that the modified protein has become, as a result of denaturation stabilization, much less homogeneous. Desiring to obtain additional data on this question (with determination under more standardized conditions and with narrower ranges of salt concentration), we selected the interesting method, proposed by Zelensky [4], and obtained salting-out curves of our preparations, i.e., of crystalline egg albumin and its modified (stabilized) form. The experimental result supports the previously established facts and shows graphically (Figure 2) the nonhomogeneity of the protein particles, which occurs as the result of denaturation stabilization. These data undoubtedly reflect changes of structure of the protein molecules during stabilization by urea treatment.

The observed complete solubility of the modified form at isoelectric reaction, and in the presence of salts, as is characteristic of egg albumin, is evidence that the resistant (modified) protein is native, that is, that its molecules retain the type of structure, characteristic of the initial native protein.

Immunochemical Study of Modified (Stabilized) Egg Albumin

New convincing evidence of the native structure of molecules of the modified form was obtained from a study by the immunochemical method. Various preparations of stabilized protein isolated, as described above, were studied in parallel with ordinary crystalline egg albumin and with this same protein, denatured by acid. A comparison of the modified form of protein with known preparations of native and denatured protein made it possible to determine similarity to one or the other. Methods of determination, described in detail by O. V. Lobachevskaya, were published previously [5].* A feature of the method consists in the fact that it is possible to obtain data not only of qualitative, but also of relatively quantitative character. It is possible to determine not only the antiserum titer, but also the zone of greatest precipitation dependent on the concentration of protein antigen in the sample.

In the experiments, antiserums to native and to denatured (by acid) egg albumin were used. Each was tested for ability to precipitate with all three preparations enumerated above – modified, native and denatured egg albumin. The data obtained are given in Table 2 (a and b).

TABLE 2

Precipitation Reaction of Serum of Rabbits Immunized to Native Egg Albumin (a) and Albumin Denatured by HCl (b).

Test-antigens: egg albumin	Amount of protein-antigen in sample, mg									
	2.5	1.125	0.4166	0.1389	0.04627	0.0154	0.00514	0.00171	0.000571	0.000143
a										
Native	–	–	++	++++	+++	±	–	–	–	–
	+	+	++	+++	++++	++	+	–	–	–
	++	++	+++	++++	+++	++	+	±	–	–
	–	+	+	++	+++	++++	+++	++	+	–
Denatured by acid	–	–	–	–	±	–	–	–	–	–
	–	–	–	–	–	±	±	–	–	–
	–	–	–	–	–	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–
Stabilized	±	+++	+++	++	+	±	–	–	–	–
	+	++	++++	+++	++	–	–	–	–	–
	+++	++	++	++	+	+	±	–	–	–
	+++	++	++	++	+	±	–	–	–	–
b										
Native	–	–	–	±	±	–	–	–	–	–
	–	–	–	–	–	–	–	–	–	–
	–	–	±	±	+	+	±	±	–	–
	–	–	±	±	+	±	±	–	–	–
	–	–	–	–	–	±	±	±	–	–
Denatured by acid	–	–	–	+	++	+++	++++	+++	++	+
	–	–	–	+	++	++++	+++	++	+	–
	–	–	–	±	+++	++++	+++	++	±	–
	–	–	–	–	+	+++	+++	++	±	–
	–	–	–	±	±	+++	++++	+++	++	±
	–	–	–	+	++	+++	+++	++	+	–
Stabilized	–	–	±	±	–	–	–	–	–	–
	–	±	±	–	–	–	–	–	–	–
	+	–	+	–	–	–	–	–	–	–
	+	±	±	–	–	–	–	–	–	–
	–	–	±	±	±	±	±	±	–	–
	±	±	–	–	–	–	–	–	–	–

* We take the opportunity to express to O. V. Lobachevskaya deep gratitude for assistance in the present series of experiments.

The experimental results gave a clear answer to the question raised: the modified (resistant to denaturation) protein with respect to immunochemical specificity is native. Antiserums to denatured protein (Table 2, b) do not react with the modified form of egg albumin or with control native protein. They give an intense reaction with the homologous antigen – denatured (by HCl) albumin. Conversely, antiserums to native protein (Table 2, a) give intense precipitation with the modified form, and approximately to the same degree (possibly somewhat more strongly) react with their homologous antigen. Considering the high specificity and sensitivity of the immunochemical method, it should be noted that the facts obtained are important evidence for the native type of macrostructure of the molecules of the modified protein.

Sulfhydryl Group Content of Stabilized Protein

In the following series of experiments our purpose was to determine the amount of sulfhydryl groups present in the modified protein, in order to compare ordinary egg albumin and its stabilized form with respect to this property.

The modified and initial proteins were studied in the experiments at a concentration of 1.5-2.0%. Sulfhydryl groups were determined by the ferricyanide method, as usual [6]. Their content was found by means of complete denaturation of the protein, for which the maximal amount of urea was used – 1000 mg per 1 ml of reaction mixture.

In the first experiments, carried out on various protein preparations, observations indicated that the stabilized form contains considerably less sulfhydryl groups than the ordinary egg albumin (Table 3). However, in reality this was not so.

TABLE 3

Apparent Content of Sulfhydryl Groups in Modified Egg Albumin
(In %, calculated on cysteine)

Denaturation temperature in degrees	Denaturation time	Amount of SH-groups in egg albumin (control)	Amount of SH-groups in modified egg albumin
37	1 hour	0.95	0.64
37	1 hour	0.96	0.57
37	30 min.	0.93	0.49
37	1 hour	0.93	0.71
34	1 hour	0.97	0.60

TABLE 4

Presence in Native Modified Egg Albumin of Reducing Groups, Oxidized by Ferricyanide under Condisions Specific for SH-Groups •

Experimental conditions: Temperature in degrees	Experimental conditions: Time	Amount of groups reducing ferricyanide•, in %	Remarks
37	30 min.	0.20	In control experiments (with ordinary crystalline egg albumin) reduction of ferricyanide did not occur
37	1 hour	0.22	
21	1 hour	0.18	
21	30 min.	0.17	
37	30 min.	0.25	
22	20 min.	0.125	
20	1 hour	0.137	

• The amount of reducing groups is given in percent, calculated by analogy with sulfhydryl groups (and for comparison of values) on cysteine.

In several control experiments, carried out with addition of water instead of urea (i.e., without denaturation of protein), we noticed that the modified protein yielded a certain amount of free sulfhydryl groups. As is known, native egg albumin contains absolutely no free SH-groups oxidizable by ferricyanide. Thus, the observed fact was entirely unexpected. This was carefully checked, whereupon it appeared that solutions of native modified protein do not give the nitroprusside reaction, although they always reduce a certain amount of ferricyanide.

Results of a number of experiments are given in Table 4.

The absence in modified protein of the nitroprusside reaction indicates that the detectable reducing groups are not sulfhydryl groups, even though they react with ferricyanide under conditions characteristic for oxidation of SH-groups only. In a previous paper [6] we found and described similar "non-sulfhydryl" reducing groups in a peptic hydrolyzate of egg albumin. The suggestion was made (supported by experiment) that these were certain transformation products (possibly of oxidation) of tyrosine.

It is appropriate to recall these data here. It can be assumed that as a result of change in structure, occurring during prolonged treatment of egg albumin with urea, certain reducing groups are "uncovered", previously more firmly bound, and acquire the ability to reduce ferricyanide under the given conditions. Whatever the possible explanation, it can be said with assurance that the fact of the appearance in native (modified) egg albumin of free groups, reducing ferricyanide at pH 6.8, which do not exist in the ordinary protein, indicates that stabilization is associated with definite changes in structure of the molecules.

This fact compelled us to turn again to the determination of sulfhydryl group content in the stabilized form of protein. Experiments were carried out in which the amount of detectable reducing groups (sulfhydryl and others) was compared with the degree of denaturation of the modified protein mass. For this purpose parallel with determination of SH-groups (under the same conditions) in mixtures identical in composition the amount of protein transformed to the insoluble form was determined. After heating, isoelectric "precipitant" was added as usual to the mixture, and the protein precipitate was determined by the biuret reaction. The data obtained are given in Table 5.

TABLE 5

Relation between Amount of SH-Groups, Found in Modified Egg Albumin (upon Denaturation), and Degree of Denaturation

Urea – 1000 mg per 1 ml of reaction mixture (maximal amount); pH 6.8

Denaturation time	Denaturation temperature °C	Total (initial) amount of protein in mg/ml of solution	Amount of SH-groups found, calculated on cysteine, in %	Amount of protein transformed to insoluble form, in mg/ml
1 hour	37	16.2	0.36	9.24
1 hour	50	16.2	0.39	11.3
1 hour	37	11.9	0.81	8.9
30 min.	37	7.1	0.49	2.2
1 hour	37	21.3	0.55	10.9

These experiments once again showed the great resistance of the stabilized form of egg albumin. Even the maximal amount of urea (1000 mg per 1 ml of reaction mixture), usually used in the determinations, does not produce complete denaturation of the stabilized protein. A considerable part of it remains untransformed under these conditions and therefore the total content of SH-groups in the modified protein (Table 3) appears to be less than in ordinary crystalline egg albumin. The incomplete denaturation of the stabilized material is also characterized by the amount of protein transformed to the insoluble form. The ordinary unmodified protein studied in parallel was, as always under these conditions, completely denatured, and the percent of liberated SH-groups was maximal (see [1,2]).

This fact is yet another objection to the possible view of the "pre-existence" of a stable group of molecules in the protein mass. As is known, the maximal amount of urea (1000 mg per 1 ml) rapidly and completely denatures native egg albumin. If there were particles retained in the protein mass, stable to such high urea concentration, they would remain untransformed and the protein would not be fully denatured. Only by treatment with a denaturing agent is it possible to obtain material of the high stability described. It is clear that the latter is produced as a result of modification. The stable portion of molecules does not pre-exist in the protein mass (and is not "selected" in the denaturation process), but arises during treatment.

The last experiments in Table 5 show that the percent of liberated SH-groups in modified protein may exceed the amount transformed to the insoluble form. This occurs because of the fact that in modified egg albumin, besides sulfhydryl, there are also other reducing groups, which are responsible for the observed "surplus".

Thus the experiments on investigation of SH-groups in the stabilized form of egg albumin have shown: a) that its molecules are structurally different from the initial protein, since they contain in the native state a certain amount of free reducing groups, not sulfhydryl; b) that its molecules contain approximately the same amount of sulfhydryl groups as is present in ordinary egg albumin; this is additional evidence of the native state

of the particles of modified protein; c) that its molecules have much greater stability to denaturation than has any constituent part of ordinary preparations of crystalline egg albumin. These data show that the stable molecules do not "pre-exist" in the protein mass, but arise as a result of treatment with a denaturing agent, that is, as a result of modification of the initial protein.

Electrophoretic Study of Modified Form of Egg Albumin

With the purpose of establishing the difference between the modified and initial proteins (or their identity) an electrophoretic study was conducted on egg albumin stabilized by urea.

The stabilized protein for these experiments was isolated from equilibrium systems, kept at room temperature 4-10 months. In these the initial protein usually was twice recrystallized egg albumin. Only in isolated experiments was purified, but not crystalline, protein used. The amount of urea was 300-400 mg per 1 ml of reaction mixture; initial protein concentration – 7-8%. The stabilized portion of protein was isolated from the equilibrium system, as in the preceding experiments, a solution containing 1.7-2.0% modified albumin being obtained.

Electrophoresis was carried out in M/25 phosphate buffer, pH 7.2, ionic strength 0.097, potential gradient 3.5 V/cm. For comparison, ordinary crystalline egg albumin was studied under the same conditions. Several typical experiments are given in Figure 3, where the course of electrophoresis with time of various preparations is shown.

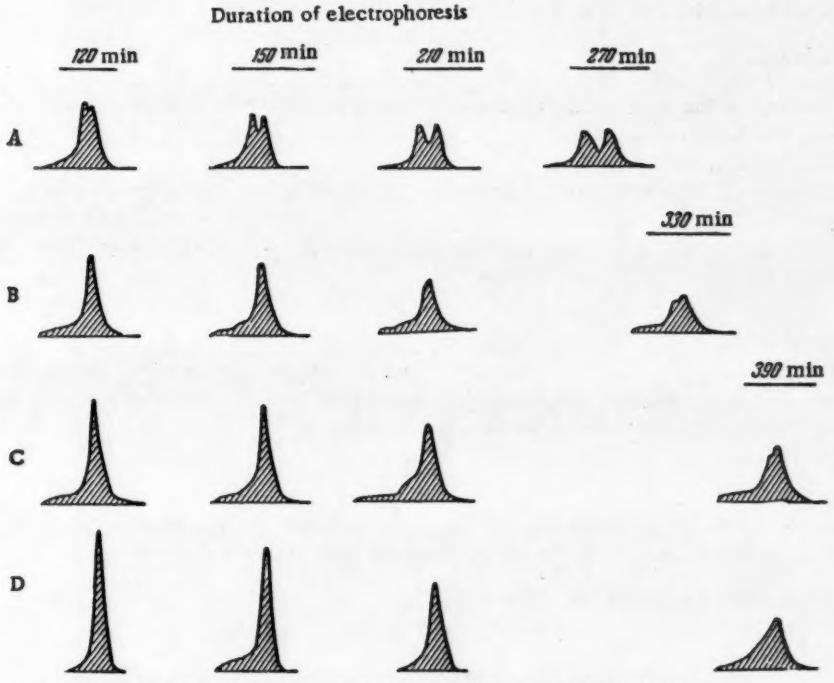

Fig. 3. Electrophoresis of crystalline egg albumin and its stabilized form obtained by action of urea:
A) crystalline egg albumin, protein concentration – 1.71%; B) stabilized fraction, isolated from an equilibrium, containing 300 mg/ml of urea, protein concentration – 1.71%; C) same, protein concentration 1.81%; D) same, but equilibrium contained 400 mg/ml of urea, protein concentration – 1.92%.

As usual ([7 1]), crystalline egg albumin during electrophoresis under the conditions described moved in the form of two peaks. Clear division of components could be observed within two hours (and even earlier) from the beginning of the experiment. Later the slow component progressively lagged behind.

A different picture was found in study of the modified (stabilized) form of egg albumin. Division of the primary peak did not occur at all in the first 2-3 hours. The presence of two main (in amount) components could be established only after $5\frac{1}{2}$ - 7 hours, during which division of peaks occurred to a small extent, although it was quite distinct (see Figure 3, B and C). In some cases even upon prolonged electrophoresis we could not detect it (for example see Figure 3, D). On the other hand almost from the very beginning of each experiment with modified protein there could be seen a small amount of third component, moving at a slower rate than the two main ones. It sometimes appeared in the form of a quite clear separate peak. In other cases it formed a characteristic slope in the corresponding position of the electrophoretic curve. From the course of the experiments the impression was created that the last, slowest "front" of particles was all the time (gradually) in small amounts being filled by molecules from the mixture of the main components. We will note a possible analogy between the third component, observed in electrophoresis, and the small amount of easily salted out fraction, which was found in preparations of stabilized egg albumin (upon saturation of the solutions with ammonium sulfate to 33 and 50%) and which is not found in the ordinary crystalline protein.

These observations show that modified (stabilized) egg albumin with respect to electrophoretic properties also is basically different from the initial protein. Although the main protein mass, like the initial preparation, consists of two chief components, yet the degree of difference between them is considerably decreased. The two components converge with respect to electrophoretic mobility. Even for an initial division they require very prolonged fractionation in the electric field, and in some cases the main mass of modified protein moves the whole time as one peak, although at the end it is very diffuse, indicating nonhomogeneity of the particles (see Figure 3, D).

These data also provide evidence of structural changes, occurring during treatment of protein with urea. This is also indicated by the presence of a certain amount of a third fraction, moving slower than the others during electrophoresis. The latter fact is in agreement with the observations on solubility, which showed that as a result of modification the protein particles become less homogeneous. In part supporting this view (the third fraction), the electrophoretic data indicate that as a result of changes occurring in the macrostructure of the particles, the electrophoretic homogeneity of the main mass has increased. It is thus possible that as a consequence of structural changes interaction has increased between particles and therefore their separation has become more difficult.

SUMMARY

From the system egg albumin – urea, which has attained a "false equilibrium" (when the denaturation process has stopped, without reaching completion [1]), protein can be obtained, which has been stabilized by the action of urea. A method has been developed for its isolation. It is based on isoelectric precipitation of the denatured portion of the protein from the system and salting out of the resistant native albumin from the remaining fraction.

The stabilized form of protein, obtained by treatment with urea, has high resistance not only to urea, but also to other, diverse denaturing agents – alcohol, potassium thiocyanate, sodium salicylate.

Solubility (ability to be salted out) of modified egg albumin is markedly different from the solubility of the initial protein. The stabilized protein is much less homogeneous. Part of it acquires the property of a globulin, being salted out at saturation with ammonium sulfate up to 0.33 and 0.50. Another (small) part acquires greater solubility, being salted out only in the range 0.67-0.77 saturation. No such fractions are contained in the initial, twice recrystallized, egg albumin. Total solubility of the stabilized form is somewhat decreased.

In immunochemical properties the modified (stabilized) protein is identical to the initial native egg albumin and differs sharply from the denatured.

The stabilized protein, a native protein, contains a certain amount (0.12-0.22%, calculated on cysteine) of free reducing radicals, oxidized by ferricyanide under conditions specific for SH-groups. However, they are not sulfhydryl, since they do not give the nitroprusside reaction. In ordinary native egg albumin there are no radicals oxidized by ferricyanide under these conditions.

However, content of SH-groups, determined by means of denaturation, is approximately the same in preparations of the initial and changed forms.

The electrophoretic properties of the modified protein are different from the properties of the initial. The usual division into two components, varying in electrophoretic mobility, is more difficult in the stabilized protein; its particles are less homogeneous; the appearance of a certain amount of third component in the preparations was noted, which was not present in the initial material.

The data obtained indicate that the molecules of the resistant (modified) protein retain the type of structure of the native egg albumin, but exhibit some structural changes by which the resistant particles are distinguished from the initial ones.

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HEAT DENATURATION OF GLIADIN

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Little work has been devoted to the problem of the denaturation of the wheat protein gliadin.

Shamshikova [1] showed the appearance of sulfhydryl groups in gliadin upon heat denaturation. Akhmatov and Pavlova [2] denatured gliadin by action of ultraviolet rays and established the resulting change in viscosity of its solutions and increase of solubility in water. Lenarsky [3] studied change in gliadin upon heating the wheat kernels. Ponomarov, Orlova and Tongur [4, 5] followed the changes in certain physicochemical properties of gliadin upon heat denaturation.

In the present paper the results are given of a study on the effect of heating upon the refraction and surface tension of gliadin solutions and on the effect of temperature and duration of heating upon the kinetics of its heat denaturation.

EXPERIMENTAL

Gliadin was obtained by a method based on its extraction with 70 % ethyl alcohol from gluten [6]. The gliadin was obtained as a white powder containing 8.5% moisture, total Kjeldahl nitrogen 17.75%, and ash 0.045%; pH of its alcoholic solution 7.14. The dry gliadin was denatured by heating in a drying chamber at a given temperature for a given time.

Determination of Refraction of Gliadin Solutions

Refraction was determined at 20° of 0.1% alcoholic solutions of the initial and heat-denatured gliadin, heated to various temperatures (from 20 to 70° with intervals of 6°).
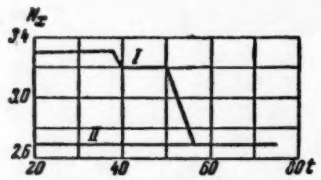
Fig. 1. Specific refraction of gliadin: I – of initial protein solution; II – of denatured protein solution; t – extent of previous heating, in degrees.

In Figure 1 is shown the relation of the specific refraction (N_x) of solutions of native and denatured gliadin to the previous heating. Calculations were made according to the equation $N_x = \frac{n-1}{d}$ (n is the coefficient of refraction; d is the density of solution). From these data it is evident that with increase of temperature the specific refraction of the native gliadin solution at first remains constant, and then drops abruptly (in the interval 40-50°). N_x of the already denatured protein solution remains constant independently of temperature.

Specific refraction increment $N_x - N_0$ (N_0 is the specific refraction of solvent) of native protein solutions also falls abruptly from 0.0027 to 0.0022 in the interval 40-50°. For solutions of already denatured protein this value (0.0022) remains constant (for human serum albumin this increment ≈ 0.0019, for egg albumin ≈ 0.0018 [7]).

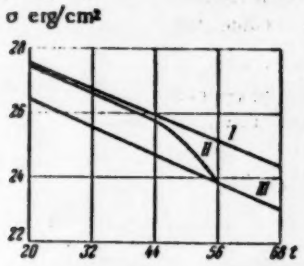

Fig. 2. Relation of surface tension to temperature: I – of 70 % alcohol; II – of 1% initial gliadin solution; III – of 1% denatured protein solution.

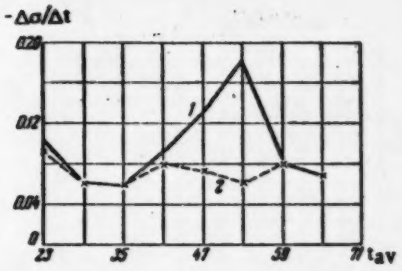

Fig. 3. Relation of thermal coefficient of surface tension to temperature: 1 – initial protein; 2 – denatured protein.

Surface Tension of Gliadin Solutions

The relation of the surface tension value (σ) to temperature of alcoholic solutions of native and denatured gliadin was established. Determinations were made by the method of maximum bubble pressure with Rebinder's apparatus. These data are given graphically in Figure 2.

From the results obtained it follows that gliadin with respect to a 70% ethyl alcohol solution is a surface-active substance. The relation $\sigma - t$ for a native protein solution shows that at 40° there apparently occurs the beginning of intramolecular changes, reflected in the surface tension value, since the curve has a break which is absent for the denatured gliadin solution. The time of holding the sample in the apparatus at the experimental temperature, 240 minutes, exerts but slight effect on the surface tension value.

From the data on temperature dependence of surface tension for solutions of native and denatured gliadin it is possible to calculate the thermal coefficient of surface tension of these solutions ($\Delta\sigma/\Delta t$). The values obtained are given in Table 1.

TABLE 1

Values of Thermal Coefficient of Surface Tension for Native and Denatured Gliadin Solutions

Temperature in degrees	Initial gliadin solution		Denatured gliadin solution	
	surface tension (σ)	$-\frac{\Delta\sigma}{\Delta t}$	surface tension (σ)	$-\frac{\Delta\sigma}{\Delta t}$
20	27.50		26.50	
		0.08		0.09
26	27.00		25.98	
		0.06		0.06
32	26.61		25.59	
		0.06		0.06
38	26.23		25.20	
		0.09		0.08
44	25.68		24.71	
		0.11		0.07
50	25.01		24.28	
		0.18		0.06
56	23.90		23.90	
		0.08		0.08
62	23.40		23.42	
		0.06		0.07
68	23.01		23.00	

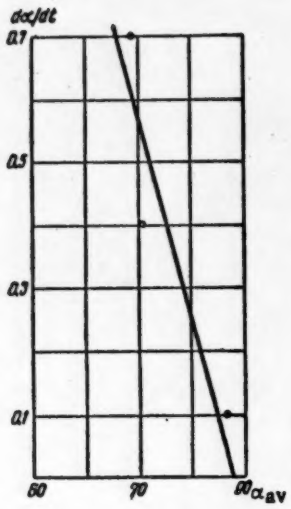

Fig. 4. Graphic calculation of α_m

In Figure 3 is given the curve for the relation of the thermal coefficient of the indicated solutions to average temperature, t_{av}.

An abrupt break in the curve at about 50° is characteristic for the native gliadin solution, which apparently indicates molecular changes occurring in the protein at this temperature, which can be considered as characteristic "critical" changes.

Kinetics of Heat Denaturation of Gliadin

During heat denaturation of protein a change occurs in a whole series of its physicochemical properties (solubility, optical activity, water-absorption capacity, swelling, etc.). Any of these features can be chosen as an index of the rate of heat denaturation.

It has been found that denaturation depends not only on temperature, but also on duration of heating, which was also confirmed by our experiments [4].

As a number of workers have demonstrated [8, 9] the kinetics of denaturation is defined by an equation analogous to the equation for a first order reaction.

It has been shown for albumin that the concentration of unchanged protein in solution is a logarithmic function of the time of heating, that is, that the degree of denaturation at any given moment is proportional to concentration of unchanged protein and to the rate constant of denaturation K = 0.00074 [10].

From experiments of Lenarský [3] on the study of kinetics of gliadin denaturation during heating of wheat kernels, it follows that the rate constant of gliadin denaturation depends both on temperature of heating the kernels and on initial moisture content, and varies little with the wheat variety.

In order to define the kinetics of heat denaturation of gliadin we determined changes in its optical activity during denaturation. For simplicity of calculation all angles of rotation are given in the first quadrant, that is $[\alpha] = 180 - [\alpha]'$. Values obtained for $[\alpha]$ are given in Table 2.

TABLE 2

Changes in Angle of Rotation of Gliadin Solutions during Denaturation

$[\alpha]_t$	Initial protein solution	Denaturation temperature in degrees			
		70		130	
		60 min.	120 min.	60 min.	120 min.
1 In degrees	90 ($[\alpha]_0$)	84	58	48	32
2 In %	100	73.8	51.0	42.2	28.2

Taking into consideration that the kinetics of heat denaturation of gliadin is defined by an equation analogous to the equation for a monomolecular reaction, and that a proportionality exists between the value $[\alpha]^\circ$ and the amount of gliadin being denatured, one can write $\frac{d\alpha}{dt} = K(\alpha_0 - \alpha_t)$. This equation is a straight line, permitting graphic determination of the value $[\alpha]_m$, the angle of rotation at the final moment of denaturation. (α_{av} and 2_0) values were calculated from data in Table 2. The value found for $[\alpha]_m$ is 88°, and $[\alpha]^\circ_m$ = 2° (Figure 4).

Expressing in % the changes in angle of rotation of a gliadin solution during denaturation from the initial, taken as 100 (Table 2, 2), the amount of gliadin remaining undenatured can be calculated.

By use of data in Table 2, the denaturation rate constant can be calculated according to the equation:

$$K = \frac{1}{t} \ln \frac{\alpha_0}{\alpha_t}.$$

TABLE 3

Rate Constant of Gliadin Denaturation.

Values	Denaturation temp. 70°		Denaturation temp. 130°	
	60 min.	120 min.	60 min.	120 min.
K	0.00512	0.00560	0.01435	0.01054
K_{av}	0.00536±5%		0.01245±7%	

The values obtained, given in Table 3, show that the rate constant of heat denaturation of gliadin changes very little with time of denaturation, but increases with temperature of denaturation.

Knowing K, it is possible to find the amount of denatured gliadin (x) and that remaining undenatured (y) at any moment of denaturation [$x = a(1 - e^{Kt})$ and $y = ae^{-Kt}$; we take a = 1], and also to calculate the time necessary for denaturation of half the gliadin, $\tau_{1/2} = \frac{\ln 2}{K}$; $\tau_{1/2}$, which for denaturation temperature 70° = = 55.6 minutes.

SUMMARY

Determination of the relation of surface tension of solutions of native and denatured gliadin to heating showed that in the interval 40-50° the beginning of intramolecular changes occurs, which were determined by measurement of surface tension.

Specific refraction and refraction increment of native gliadin solutions on heating to 40-50° fall sharply, while in heat-denatured gliadin these indexes are constant. The kinetics of heat denaturation of gliadin was studied with respect to change in optical activity of its alcoholic solutions.

It was established that the rate constant of denaturation at 130° is greater than at 70°. With twofold increase of denaturation temperature the denaturation is accelerated approximately twofold. The time for denaturation of half the gliadin was found.

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EFFECT OF NUCLEIC COMPOUNDS ON ACTIVITY OF PROTEOLYTIC ENZYMES OF C. DIPHTHERIAE

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Nucleic acids, which are high polymer compounds, are contained in abundance in cells where intensive protein synthesis occurs. Especially rich in these substances are yeasts and bacteria [1]. Nucleic acid content in bacteria may in certain cases reach more than 15% of the dry weight of the bacteria [2].

Belozersky et al. [3], studying nucleic acids of C. diphtheriae at different growth stages, showed that in aging of a culture and slowing of its growth the amount of RNA is sharply reduced. Caldwell, Mackor and others [4] in a study of growth of Bacillus lactis aerogenes, determined that the amount of RNA in bacterial cells is proportional to the growth rate. Gale and Folkes [5] in a study of nucleic acid content of Staphylococcus aureus in relation to protein synthesis note that with culture on a synthetic medium and with addition to this medium of purine and pyrimidine bases there occurs marked increase in synthesis of protein and nucleic acids. The authors established that with reduction in nucleic acid content in the cell there occurs a sharp fall of protein synthesis; hence they deduce an interrelation between nucleic metabolism and protein synthesis. Davidson, Brachet, Kedrovsky and others [6-8] consider that protein synthesis occurs in the cytoplasmic granules, rich in both high-polymer phosphorus compounds, and a large quantity of enzymes in the form of phosphatases, peptidases and proteases, which also apparently participate in synthesis of peptides and proteins.

In this connection it is of interest to note the work of Slavik and Smetana [9] on the effect of ribonucleic acid (RNA) on activity of trypsin, papaine and cathepsin of liver. It was shown that yeast RNA, which inhibits hydrolysis of proteins and peptides, does not block the transpeptidase reaction. On the basis of the experimental data, the authors suggest that nucleic acids may participate in regulation of enzyme reactions in the cell. Sorm and Hrubesova made studies in the same direction [10]. It was determined that pancreatic RNA blocks activity of pancreatic proteases.

It is evident from the literature data presented that the role of nucleic acids in the process of protein synthesis still remains unclear. It is possible that they act as phosphate donors or are a factor, in a specific way directing enzyme activity in the processes of protein synthesis. It may be suggested that nucleic acids participate directly in protein synthesis as a source of energy, which is liberated from phosphate bonds with participation of the appropriate enzymes.

METHODS

For study of the effect of nucleic compounds on activity of proteolytic enzymes of C. diphtheriae, nucleoproteins and RNA, obtained by us from a two-day culture of C. diphtheriae strain PW 8 by the method of Belozersky [11], and sodium nucleate (of yeast, Merck) were used. Properties of these preparations are given in the table. Concentrations of nitrogen and phosphorus are calculated for an absolutely dry and ashless substance.

SETUP OF ENZYME EXPERIMENTS

From a two-day culture of C. diphtheriae after washing with physiological solution and careful grinding with powdered glass a 10% bacterial suspension in phosphate buffer was made.

Properties of Nucleic Compounds

Name of preparation	N in %	P in %	Orcinol reaction	Diphenyl-amine reaction	Biuret reaction
RNA					
C. diphtheriae	16.5	8.5	+	–	–
Nucleoprotein					
C. diphtheriae	9.65	0.95	+	very weak	+
Sodium nucleate	13.35	8.5	+	–	–

Autolysis was conducted at pH 7.0 and 8.0. A mixture of 4 ml bacterial suspension, 10 ml buffer solution and 2.5 ml of nucleic compound (2% solution of RNA, nucleoprotein or sodium nucleate) was diluted with distilled water to a total volume of 20 ml and incubated 24 hrs at 35°.

Toluol was added as preservative. The same mixture served as control, but without addition of nucleic compound.

After autolysis the proteins were precipitated with 20% CCl_3COOH and the filtrate was divided into two parts. In one, residual nitrogen was determined by the Kjeldahl micromethod. Calculations were made, after deduction of nitrogen of the respective controls, for an absolutely dry substance. The second part of the filtrate was used for analysis by two-dimensional chromatography according to Sisakyan, Bezinger and others [12]. Trichloroacetic acid was removed from the filtrate by extraction four times with ether, after which the liquid was concentrated on the water bath. From the residue the free amino acids were extracted with acid acetone (1 ml 10 N HCl per ml of acetone). From the acetone extract, concentrated by very mild heating, hydrochloric acid was removed under vacuum. The residue was dissolved in 1 ml distilled water and in this solution the total nitrogen was determined by the Kjeldahl micromethod. After setting up a number of preliminary chromatograms the amount of protein nitrogen (260-300 μg) was found, necessary for the clearest development of the amino acid spots. Leningrad No. 2 paper was used for chromatograms.

RESULTS

In the study of the effect of nucleic compounds on activity of proteolytic enzymes of C. diphtheriae there was noted an almost threefold reduction in residual nitrogen content in experimental samples upon addition of nucleoprotein and RNA in comparison with the control (Figure 1).

An entirely different picture was observed with addition of sodium nucleate; in nearly all experiments at pH 7.0 an increase of residual nitrogen occurred. This is apparently related to the fact that sodium nucleate, although similar in nitrogen and phosphorus content to RNA of C. diphtheriae (table), yet in all probability differs from it in chemical structure and specific action.

In the literature there are indications of the fact that addition of an oxidizing agent in the form of hydrogen peroxide [13, 14] in the study of activity of proteolytic enzymes was accompanied by increase of the synthetic function of proteases.

We also added hydrogen peroxide during autolysis of samples with nucleic compounds. In view of the high activity of catalase of C. diphtheriae the addition of hydrogen peroxide to samples was made extremely slowly. It was found that hydrogen peroxide caused almost no changes in activity of proteolytic enzymes of C. diphtheriae at pH 7.0

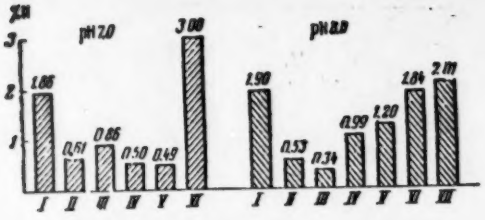

Fig. 1. Change in activity of proteolytic enzymes of C. diphtheriae under the influence of nucleic compounds: I – bacterial suspension (control); supplements: II – nucleoprotein, III – nucleoprotein + H_2O_2; IV – RNA; V – RNA + + H_2O_2; VI – Na nucleate; VII – Na nucleate + + H_2O_2.

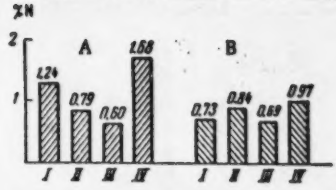

Fig. 2. Blockage of proteases of C. diphtheriae in the presence of nucleic compounds.
A – experiments without protease inactivation; B – experiments with protease blockage by o-hydroxyquinoline; I – bacterial suspension (control); supplements: II – RNA; III – nucleoprotein; IV – Na nucleate. Autolysis 24 hrs., pH 8, t – 37°.

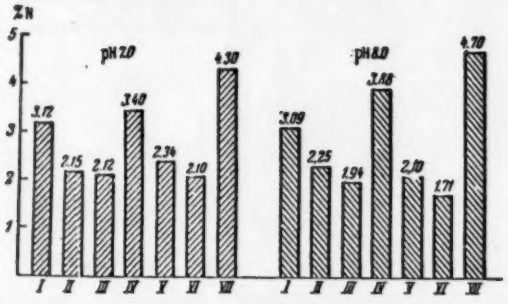

Fig. 3. Change in activity of proteolytic enzymes of C. diphtheriae under influence of nucleic compounds: I – bacterial suspension + amino acid mixture; supplements: II – nucleoprotein; III – RNA; IV – Na nucleate; V – nucleoprotein + H_2O_2; VI – RNA + H_2O_2; VII – Na nucleate + H_2O_2.

In experiments at pH 8.0, the most marked changes were observed under the influence of nucleoprotein. We noted analogous results with RNA. With respect to experiments with sodium nucleate, almost no changes in nitrogen content, in comparison with controls, were found.

Thus, the experimental data indicate that upon addition to a bacterial suspension of nucleoprotein or RNA there occurs sharp reduction of residual nitrogen; upon addition of Na nucleate, reduction of nitrogen is not observed.

For investigation of the problem: to what is the reduction of residual nitrogen related under these conditions and is it a consequence of the changed direction of protease activity under the influence of nucleic compounds [9, 10], we set up experiments with inactivation of proteolytic enzymes of C. diphtheriae by o-hydroxyquinoline (0.004 M).

From Figure 2 it is seen upon blockage of proteases by o-hydroxyquinoline in the presence of nucleic compounds no marked changes in residual nitrogen content were observed in comparison with the control. In analogous experiments without protease inactivation marked reduction in residual nitrogen did occur.

The question of what substances are formed at the expense of the reduction in residual nitrogen in experiments with addition of nucleic compounds, remains for the present unclear.

In connection with the changes found in residual nitrogen, in order to investigate the synthetic function of proteases under the influence of nucleic compounds experiments were set up in which an amino acid mixture was added to the bacterial suspension, in addition to the usual supplements.

The amino acid mixture was prepared with the consideration that it should resemble the amino acid composition of muscle globulin. For this purpose the following amino acids were taken: D-glutamic, aspartic, alanine, phenylalanine, tyrosine, cystine, methionine, histidine, lysine, arginine, threonine, serine, valine,

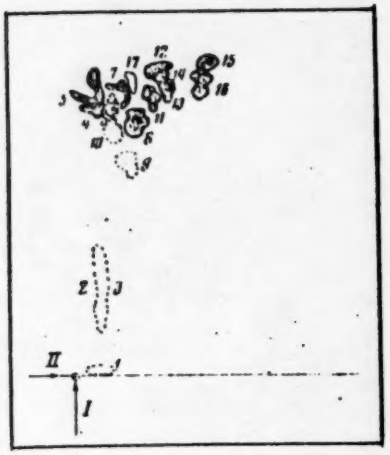

Fig. 4. Control chromatogram of amino acid mixture and bacterial suspension: 1 – cystine; 2 – arginine; 3 – lysine; 4 – aspartic acid; 5 – glutamic acid; 6 – unidentified acid; 7 – alanine; 8 – serine; 9 – histidine; 10 – glycine; 11 – tyrosine; 12 – valine; 13 – methionine; 14 – threonine; 15 – leucine; 16 – phenylalanine; 17 – proline

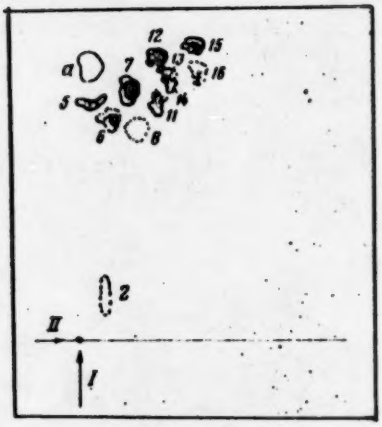

Fig. 5. Chromatogram of mixture: bacterial suspension + amino acid mixture + nucleoprotein after autolysis: 2 – arginine; 5 – glutamic acid; 6 – unidentified acid; 7 – alanine; 8 – serine; 11 – tyrosine; 12 – valine; 13 – methionine; 14 – threonine; 15 – leucine; 16 – phenylalanine; a – new spot.

leucine, proline and glycine.

The experimental results are given in Figure 3.

As is evident from the experimental data presented, here too a uniform reduction in nitrogen content was observed, in comparison with controls; it should be noted that nucleoprotein and RNA caused the reduction in residual nitrogen.

With respect to the effect of Na nucleate, in these experiments an increase in amount of nitrogen was noted; addition of hydrogen peroxide under these conditions caused a sharp increase in residual nitrogen of 38% at pH 7.0 and of 50% at pH 8.0, which indicates intensification of the hydrolytic function of C. diphtheriae proteases. Results of these experiments also indicate that under the influence of nucleoprotein and RNA, isolated from C. diphtheriae, there apparently occurred a change in activity of proteolytic enzymes of C. diphtheriae in the direction of increase of their synthetic function. In several experiments with addition of amino acid mixture, chromatographic analysis was carried out.

In Figure 4 is given the control chromatogram of the amino acid mixture. From the chromatograms (Figures 5, 6 and 7) it is seen that in all experiments a new spot (a) over glutamic acid appears and disappearance of aspartic acid occurs under the influence of RNA and of nucleoprotein; in experiments with Na nucleate traces of it are visible.

Of the sixteen amino acids used in the experiments, as is seen from the chromatograms, we noted complete disappearance of histidine, proline, lysine, cystine and in addition intensification of an unidentified spot [6] was observed.

Together with these changes in all experiments there occurred sharp reduction in glutamic acid content. As seen on the chromatograms, under these conditions there was observed marked decrease of tyrosine, methionine and serine in comparison with the control amino acid mixture.

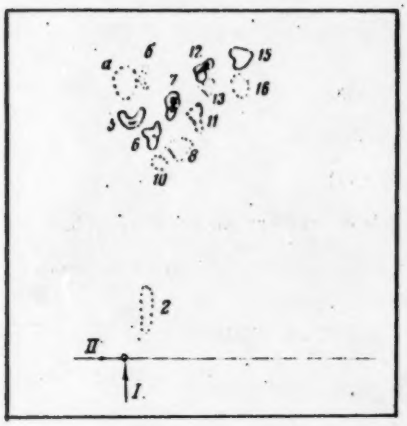

Fig. 6. Chromatogram of mixture: bacterial suspension + amino acid mixture + RNA: numbering of spots see Fig. 4; a and b – new spots.

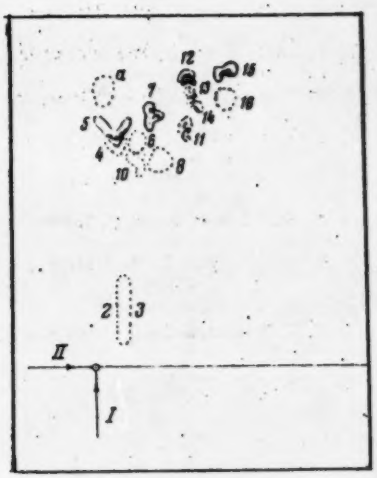

Fig. 7. Chromatogram of mixture: bacterial suspension + amino acid mixture + Na nucleate: numbering of spots see Fig. 4; a – new spot.

In addition to the changes indicated, in Figure 6 must be noted the disappearance of threonine, the sharp decrease in concentration of methionine and glycine and the appearance of two spots (a and b) over glumatic acid, the latter not being identified. Features of the experments with nucleoprotein were: complete disappearance of glycine, increase in alanine concentration and decrease in amount of methionine, threonine and tyrosine.

In experiments with sodium nucleate (Figure 7) aspartic acid is retained, and sharply decreased are concentrations of serine, glycine, tyrosine, methionine, threonine and phenylalanine.

The experimental results of study of change in composition of amino acids under the influence of nucleic compounds support our data on study of changes in amount of residual nitrogen.

On the one hand, the experimental data presented indicate that addition of nucleic compounds in the form of RNA and nucleoprotein of C. diphtheriae apparently allows a change in orientation of the activity of proteolytic enzymes of C. diphtheriae in the direction of increase in synthetic function. On the other hand, they indicate a certain specificity of action of the nucleic compounds. According to the experimental data presented above, under the influence of sodium nucleate (of yeast) under these conditions an increase in hydrolytic function of proteolytic enzymes of C. diphtheriae was observed, whereas increase in synthetic function occurred only upon participation of the nucleic compounds of C. diphtheriae.

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ISOLATION OF INTRACELLULAR GRANULES BY SEDIMENTATION IN A SEPARATOR THROUGH AUXILIARY LIQUID LAYERS OF DIFFERENT DENSITIES

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Isolation of intracellular components – nuclei, mitochondria, microsomes, and also viruses, is necessary for the solution of many important problems in modern biology and medicine. The present communication describes a new fractionation method used for isolation of intracellular granules and viruses from normal and tumorous tissues.

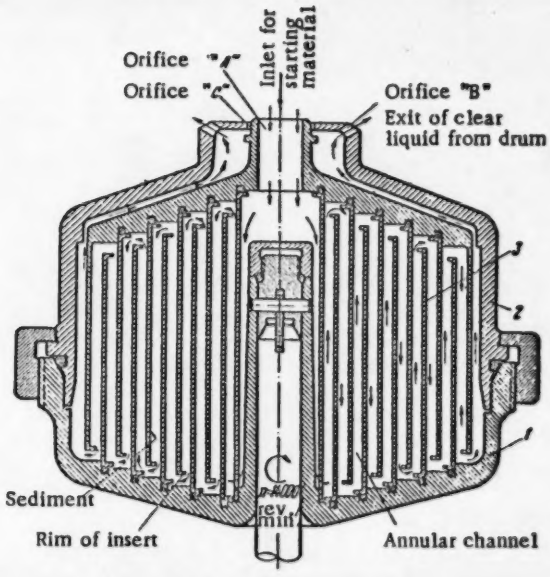


Fig. 1. Scheme of separator drum:
1) Drum base, 2) drum lid; 3) cylindrical inserts.

Tissue homogenates are usually fractionated by means of differential centrifugation [1]. An improved fractionation method has recently been proposed – centrifugation in a density gradient, whereby fractions can be obtained homogeneous in density [2,3] or in sedimenation rate [4], and which avoids multistage fractionation. However, this method is of limited application for studies of intracellular granules (mitochondria, microsomes) and viruses owing to the extremely small amounts of material obtained.

Use has been made of separators with conical inserts ASG-1-A and ASL-2 for obtaining mitochondria and microsomes in sufficient quantities for thorough investigation [5,6] but with these separators fractions can be obtained only by consecutive sedimentations. For fractionation of normal and tumor tissue homogenates, the Separator Laboratory of the Scientific Research Institute for Chemical Machinery Building has constructed a special separator type SK, intended for isolation of the intracellular granules with their simultaneous fractionation in the same drum. The scheme of the separator drum which carries out this technological function is shown in Fig. 1.

The suspension to be separated enters the drum in a continuous stream through the orifice A and passes consecutively through three annular channels formed by concentric inserts in the drum (the direction of liquid flow is indicated by arrows in the scheme). The sediment of the particles from the separated suspension settles on the inner surfaces of the inserts. In order that a suspended particle should be deposited on the insert, the condition L = S must be satisfied, where L is the path of the particle under the action of the centrifugal force, and S is the distance between the particle and the surface of the insert. If the condition L = S is not satisfied, the particle will pass into the annular space of the next insert.

The centrifugal force acting on a particle increases as the particle moves further away from the drum axis. The conditions for sedimenation of the particle are more favorable at the next insert, as not only is the speed of the particle increased by the action of the centrifugal force, but its time in the annular channel is greater.

Creation of "Density Barriers" in the Drum

It is known that the surface of a liquid in a rotating hollow closed cylinder assumes the form of a parabola. As the rotation speed of the cylinder increases, the centrifugal force becomes greater and the apex of the parabola descends, reaching infinity at sufficiently high values of the centrifugal force, when the force of terrestrial gravity may be neglected. The surface of the liquid then becomes cylindrical.

Each insert in the drum is a hollow cylinder, with its rims serving as retaining surfaces (Fig. 1). Thus, an annular layer of liquid the thickness of which corresponds to the height of the rim can be established on the drum insert. If the density of the liquid being separated is less than the density of the liquid in the annular layer, the latter can serve as a stable barrier between the liquid being separated and the surface of the insert on which the particles are being deposited.

In the present work; the annular layers (density barriers) were made with the use of sucrose solutions of different concentrations (d = 1-1.3 g/ml) while the homogenate was always made in 8% sucrose solution (d = 1.035 g/ml).

The iodine – starch color reaction was used to confirm the possibility of creation of density barriers at different inserts in the drum of the SK separator. Narrow strips of filter paper impregnated with starch paste made in 50% sucrose solution, and moistened with sucrose solution of a concentration corresponding to that of the given barrier, were attached to the inner surface of each insert. Instead of the separation liquid, 200 ml of 8% sucrose solution faintly colored with iodine was passed through the drum at a rate not below 7-8 liters per hour. The basis for selection of this rate was the high sensitivity of the reaction and the possibility of diffusion of iodine into the "barrier". To displace the iodine solution, 400-500 ml of 8% sucrose solution was passed through the drum at the same feed rate. The complete absence of the iodine – starch reaction at the inserts where density barriers had been created, and the formation of color at control inserts where there was no barrier (the rim of insert XI was cut down at several points, making the creation of a "barrier" at this insert impossible), confirmed the possibility of separation through layers of auxiliary liquid of different densities.

The concentrations of the annular liquid layers should be chosen in accordance with the densities of the particles of the suspension to be separated. For fractionation of suspensions, the inserts nearer to the rotation

axis of the drum should have "barriers" of the heaviest liquids, with a gradual decrease of density toward the periphery. This distribution of barriers of different densities prevents the sedimentation of particles of low density at the first inserts of the drum.

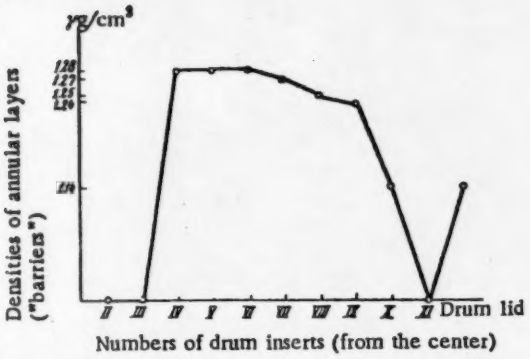


Fig. 2. Distribution of density "barriers" on drum inserts.

Barriers of different densities were created by pouring the following solutions of sucrose into the drum in the sequence: 1) 200 ml, d = 1.05 g/ml (before the separator was switched on, through orifice C); 2) 190 ml, d = 1.28 g/ml through A with the drum rotating; 3) 80 ml, d = 1.17 g/ml through A with the drum rotating. The density of the established barriers was determined experimentally by means of the iodine – starch reaction.

In order that the conditions in which the densities of the barriers were determined should completely correspond to operating conditions with homogenates, before addition of the iodine solution 800 ml of 8% sucrose solution (d = 1.035 g/ml) was passed through instead of the homogenate; this was followed by the solution colored with iodine, which was then displaced by a similar solution without iodine.

TABLE 1

Intensity of the Iodine – Starch Reaction at Different Inserts of the Drum.
Instead of homogenate, 800 ml of sucrose solution with density 1.035 g/ml was put into the drum at a feed rate of 1 liter/hour. Amount of iodine-tinted sucrose solution – 200 ml, solution for displacement of iodine, 400 ml. Feed rate of these solutions, 7 liters/hour.
++ maximum starch color, + weak color, +– traces of color; – absence of color

Densities of iodine-tinted sucrose solutions and of solutions passed to displace iodine, in g/ml	Iodine – starch reaction, number of inserts (counting from center)										
	II	III	IV	V	VI	VII	VIII	IX	X	XI	Drum lid
1.04	++	++	–	–	–	–	–	–	–	++	–
1.10	++	++	–	–	–	–	–	–	–	++	–
1.15	++	++	–	–	–	–	–	–	+	++	+
1.17	++	++	–	–	–	–	–	–	+	++	+
1.20	++	++	–	–	–	–	–	–	++	++	++
1.24	++	++	–	–	–	–	–	+–	++	++	++
1.26	++	++	–	–	–	–	+	++	++	++	++
1.28	++	++	+–	+–	+–	+	+	++	++	++	++

The results of the experiments are shown in Table 1 and Fig. 2. Fig. 2 shows that stable barriers are established from the IVth insert onward.

The explanation for the positive iodine – starch reaction on inserts II and III is that insert III is at the same vertical level as the liquid outlet in the drum. If this orifice is brought nearer to the axis of rotation of the drum, the stability of the barrier on insert III increases. By variations of the quantitative proportions and concentrations of the solutions added, it is possible to set up a great variety of density barrier distributions on the drum inserts.

The Process of Particle Sedimentation in Separation Through the Density Barriers

Four different cases of particle sedimentation can be visualized.

Case A. The density of the particle A (γ_1^A) is considerably greater than the density of the dispersion medium (γ_2) and greater than the density of the established layer or barrier (γ_3) ($\gamma_1^A >> \gamma_2$ and $\gamma_1^A > \gamma_3$); the particle A rapidly reaches the barrier, passes through it, and is deposited on the inner surface of the cylindrical insert in question.

Case B. $\gamma_1^B > \gamma_2$, but $\gamma_1^B < \gamma_3$; the particle B is able to traverse the distance to the barrier, but cannot penetrate into the layer of heavy liquid and is carried by the stream into the annular space of the next drum insert.

Case C. γ_1^C is slightly greater than γ_2; the particle C does not have time to traverse the distance S and is carried by the stream into the annular space of the next insert.

Case D. $\gamma_1^D >> \gamma_2$, but the diameter of the particle D (d^D) is considerably less than the diameter of particle A (d^A) ($d^D << d^A$); particle D does not have time to reach the layer of heavy liquid and is carried over into the annular space of the next insert.

By creating the appropriate conditions at the following inserts, consecutive sedimentation of particles B, C, and D may be effected.

The Separator Laboratory of the Sci. Res. Inst. for Chemical Machinery Building has designed, and is now building, the special separator SK-1, in which it is easily possible to set up any desired "density barriers" at the cylindrical drum inserts.

The described method has fully justified itself in the fractionation of normal and tumor tissue homogenates and has made it possible to separate particles differing both in size and in density.

The method simplifies the procedure, shortens the treatment time of the material and, which is most important, yields a large number of fairly pure fractions.

Isolation of Intracellular Granules from Rabbit Liver

The above method has been used to obtain four fractions from rabbit liver; these were subjected to analysis by electron microscopy and by biochemical methods.

Preparation of material and fractionation. The liver of a rabbit, removed immediately after killing, was washed with cold isotonic sucrose solution through the posterior vena cava until the blood had been completely removed. After the blood had been washed out, the gall bladder was removed. If the liver was not used for the experiments immediately, it was frozen and stored at − 40°. The fresh or thawed liver (50-75 g) was ground in two stages in a Waring homogenizer in ten times its own volume of isotonic sucrose solution. The nuclei must not be damaged during the homogenization; this was checked by means of a phase contrast microscope.

The nuclei and undamaged cells were separated in a centrifuge at 470 g • with a liquid layer 9 cm high in the centrifuge tube, in 5-6 minutes.

The supernatant liquid was carefully decanted through one layer of gauze; it was used as the starting material for fractionation in the separator.

"Barriers" of various densities were set up on the drum inserts by the method described above, and the material was then separated at 14,000 r.p.m. at a flow rate of 1 liter/hour. The constant flow rate was maintained by the use of glass tubes of definite diameter for connecting the vessel with the material to the separator inlet.

The last portion of homogenate remaining in the drum was displaced by an isotonic sucrose solution (300 ml) at the same feed rate (1 liter/hour). The separator was then left running for 1 hour 45 minutes to enable the particles reaching the high density barrier to reach the surface of the insert and to form a dense deposit. The deposits were washed off the inserts into isotonic sucrose solution. All the operations were carried out at 0-4°.

• In calculations of the centrifugal force the radius from the axis of rotation to the center of the centrifuge tube was taken.

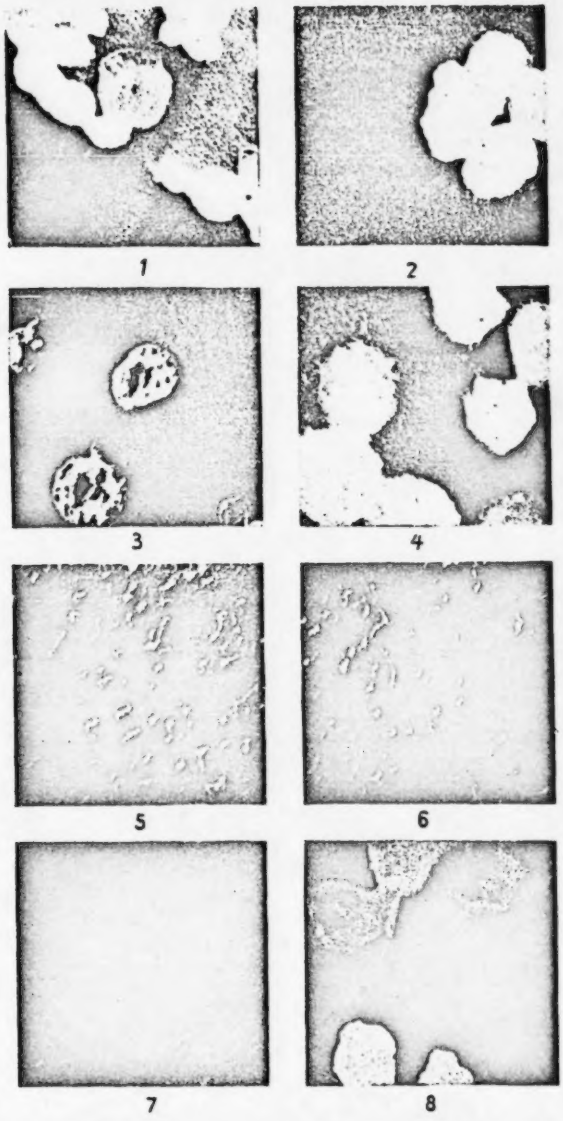
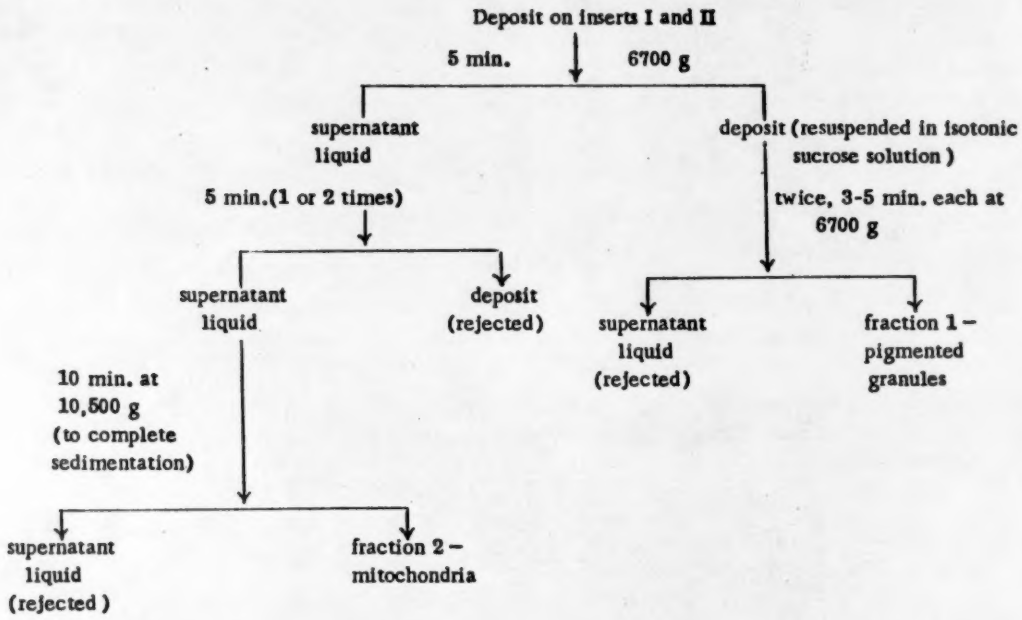


Fig. 3. Electron micrographs of rabbit liver fractions: 1 and 2) Pigmented granule fraction [in center of 1, mitochondrial wall (x 10,000)]; 3 and 4) mitochondria fraction (x 1,000); 5 and 6) microsomes (x 10,000); 7) glycogen particles (x 10,000); 8) mixture of mitochondria and pigmented granules after treatment with distilled water (X 10,000).

Distribution of deposits on the inserts. The first drum insert serves to direct the flow and does not contain a deposit. A dense dark brown deposit, which we termed fraction 1, was formed on insert II. Insert III had a deposit of a yellow-greenish color, fraction 2. Fraction 3 was a milk-white deposit found on inserts V-VIII. Finally, all the last inserts had a pink deposit, fraction 4.

Fractions 1 and 2 consisted of large granules, clearly visible in the phase contrast microscope, and fractions 3 and 4 consisted of submicroscopic particles. The first two fractions were somewhat contaminated with each other, and could be completely separated by the following scheme.


Deposit on inserts I and II
5 min.
6700 g
supernatant liquid
deposit (resuspended in isotonic sucrose solution)
5 min.(1 or 2 times)
twice, 3-5 min. each at 6700 g
supernatant liquid
deposit (rejected)
supernatant liquid (rejected)
fraction 1 – pigmented granules
10 min. at 10,500 g (to complete sedimentation)
supernatant liquid (rejected)
fraction 2 – mitochondria

Characteristics of the fractions. The nature and the purity of the fractions were studied by electron microscopy and biochemical analysis.

The specimens for electron microscopy were prepared from suspensions of the granules in isotonic sucrose solution after fixation in osmic acid for 50-60 minutes (except fraction 3, the particles in which are destroyed in fixation). A drop of the suspension was placed on the membrane and quickly shaken off. After slight drying the preparation was washed with 1-2 drops of distilled water and dried in a desiccator. Nichrome was used for the shadowing. Fig. 3 shows electron micrographs of all the fractions.

Data on ribonucleic acid (RNA) contents and succinic dehydrogenase activities of the fractions are shown in Table 2.

In an attempt to determine RNA in fraction 3 it was found that the material was completely soluble in 5% trichloroacetic acid to form an opalescent solution, and was reprecipitated on addition of an equal volume of alcohol. This precipitate was easily soluble in water. On addition of a solution of iodine in KI to this solution a specific red color was formed, which disappeared on warming and reappeared on cooling (glycogen reaction). It should also be noted that fraction 3 vanished completely if the animal had been starved for two days. All the above properties and reactions indicate that fraction 3 was glycogen.

As Figure 3 and the data in Table 2 show, fraction 1 differs from mitochondria. The completely washed fraction contains almost no succinic dehydrogenase and is stable in distilled water (Fig. 3, 8). It is interesting to note that this fraction contains considerably more iron than the other fractions (qualitative test). This gives grounds for classifying it with the iron-containing pigments of the liver. A very low RNA content and slight enzyme activity are found only in completely washed fractions of the pigmented granules. A small admixture of fraction 2 sharply raises its RNA content (for non-starved rabbits) and its relative enzymatic activity.

Fraction 2 has a group of typical characteristics of mitochondria: maximum succinic dehydrogenase activity, a specific morphology including the presence of walls, and susceptibility to lysis in distilled water.

TABLE 2

RNA Contents and Succinic Dehydrogenase Activities of Rabbit Liver Fractions.
RNA in μg/mg N; activity of succinic dehydrogenase as % of maximum activity.

Fractions	RNA (by Schneider's method [7] without extraction of lipids)	Succinic dehydrogenase activity (by Thunberg's method with a suspension of freshly prepared granules)
1	Traces	16.5
2 Normal animal	346	100
3 Animal after 2 days fasting	85	100
4	407	10

It is interesting to note that the RNA content of mitochondria decreases considerably after starvation of the animals (by a factor of 3.5 after two days of starvation). A similar effect has recently been reported by Laird et al. [8].

Fraction 4 consists of microsomes, as indicated by the particle size, high RNA content, and low succinic dehydrogenase activity. In contrast to mitochondria, the RNA content of this fraction does not change in starved animals.

Thus, by means of the density barrier separation method it proved possible to carry out simultaneous sedimentation and separation of pigmented granules, mitochondria, glycogen, and microsomes.

It is evident that this fractionation method has clear advantages both over the differential centrifugation method and over fractionation in a density gradient.

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ORGANIC ACID FORMATION IN GERMINATION OF LEGUME SEEDS

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Germination of seeds of cereals, e. g. wheat and corn, is accompanied, as our studies have shown [1, 2], by intense formation of organic acids. Together with malic and citric, aconitic acid accumulates in considerable amount; it represents up to 60% of the acids contained in sprouts. Of interest was a study of acids of the seeds of another large group of seed crops, the legumes. This group of plants, as is known, differs sharply from the cereals not only in chemical composition of the seed, but also in biological features of the nitrogen nutrition.

The organic acids of leguminous plants have been little studied. In the seeds citric acid has been found [3, 4]; in leaves of kidney bean (haricot) and bean, according to studies of Chesnokov [5], it represents up to 13.5% of the dry weight of the plant.

For more detailed study of the acid composition of the principal legumes we made a series of analyses of seeds of kidney bean, bean and pea, and also carried out experiments on study of acid formation during seed germination.

EXPERIMENTAL

Analyses for organic acid content of seeds, sprcuts and leaves of legumes were made by the technique developed by us for the cereals. In all cases the acids were obtained from dried, powdered material by prolonged extraction with ether, first without acidification, and then after acidification of the powder with sulfuric acid. The acids were separated in the form of barium, lead, and calcium salts; citric acid was determined by conversion to pentabromoacetone, and malic acid by the method of Pucher and Vickery [6].

Organic Acids of Legume Seeds

For analysis were used seeds of kidney bean variety "Saksa", of broad bean (horse bean) and of pea variety "Rostovsky". For each analysis seeds were ground to a powder, weighing ~100 g. Analytical results are given in Table 1.

The amount of free organic acids in legume seeds is insignificant: 0.006-0.007% in kidney bean and bean, 0.08% in pea. Acids are present chiefly in the form of salts. Kidney bean seeds are distinguished by the highest content; of the acids citric predominates, representing in seeds of kidney bean and bean about one-half of the total amount of acids. Malic acid represents more than 10% in seeds of kidney bean, and in bean and pea up to 5-8% of the total acid content. Volatile acids represent only about 10%. The amounts of citric acid found by us in legume seeds about coincides with data of other workers [3, 4].

Organic Acid Formation in Germination of Legume Seeds*

For experiments were used seeds carefully selected as to size and weight of kidney bean variety "Saksa", broad bean and pea variety "Rostovsky".

A portion of the seeds after grinding was analyzed for organic acid content, the second portion was planted in earth in seed boxes, located in a well lighted hothouse. Two-week sprouts were cut at a level 1 cm from the

* Student A. M. Kopina participated in this part of the work.

TABLE 1

Organic Acids of Legume Seeds.
In % of air-dried wt.

Plant	Total acids	Volatile	Citric	Malic	Unknown
Kidney bean	1.93	0.21	1.22	0.270	0.233
Bean	0.90	0.09	0.48	0.047	0.283
Pea	0.82	0.14	0.29	0.067	0.323

TABLE 2

Organic Acid Formation in Germination of Legume Seeds.
In grams per 1000 seeds and sprouts.

Plant	Total acids	Of them			
		volatile	citric	malic	unknown
Kidney bean					
Seeds	5.54	0.55	3.18	0.71	1.10
Sprouts	22.86	0.91	1.40	6.01	14.54
Bean					
Seeds	10.64	1.06	5.74	0.55	3.29
Sprouts	20.33	0.84	2.23	15.25	2.01
Pea					
Seeds	2.10	0.42	0.74	0.16	0.78
Sprouts	19.96	0.61	0.71	8.42	10.22

soil, fixed with dry heat and dried at 60-70°, then ground in a mill. Sprouts were 15-18 cm high with 5-6 leaves. Analytical results, calculated for 1000 seeds and sprounts, are given in Table 2.

Weight of powder from seeds was 100 g, corresponding to 382 seeds of kidney bean, 93 of bean and 453 of pea.

Weight of powder from sprouts was about 35 g. This amount was given by 80 sprouts of bean, 148 of kidney bean and 133 of pea.

Comparison of results of analysis of seeds and sprouts of legumes shows that germination is characterized by very intense new formation of organic acids; the total amount in kidney bean and bean increases 2-4 times, and in pea even 9 times.

The citric acid, contained in the seeds, is actively consumed, especially in kidney bean and bean, where its amount is reduced to less than half. Malic acid is formed exceptionally actively and accumulates in the sprouts; in pea and bean the amount increases 28-53 times in comparison with the initial seed content.

The content of unknown acids increases 13 times. The amount of volatile acids increases but slightly. Content of free acids in sprouts is 0.05-0.2%.

DISCUSSION OF RESULTS

The two principal groups of seed crops, cereals and legumes, differ in chemical composition not only with respect to amount and properties of protein substances and carbohydrates, but also with respect to composition of the organic acids in both seeds and leaves.

In wheat seeds the organic acid content varies within the limits 0.2-0.8%, of which more than one-half is acetic acid; citric and malic acids together represent only 5-10% [1].

In seeds of kidney bean, bean and pea there are more acids than in cereal seeds (0.8-2.12%). The proportion of acids also varies: in legumes volatile acids represent only ~10%; citric acid, contained in wheat seeds in the amount of 0.035%, in kidney bean reaches 1.22% and in bean 0.48%, that is, more than half the total acid content.

The sprouts of cereals and legumes also differ appreciably in amount and composition of acids. In cereal sprouts, aconitic acid accumulates in considerable quantities, representing up to 60% of all the acids, while in legume sprouts this acid is not found even in traces.

Citric acid in leaves of legumes accumulates in considerable quantities, up to 4-5% of the dry substance, representing more than 40%, whereas in cereals it represents only 1-2% of the acids present.

With respect to content of citric and aconitic acids the leaves of cereals and legumes are antipodes – maximal content of aconitic acid in the former and absence in the latter compensated by a high content of citric acid. This difference of cereals in content of acids genetically closely related is probably due to special features of the acid metabolism. Aconitase, an enzyme which converts cis-aconitic and isocitric acids to citric, possibly participates in this metabolism [7-9]. In the equilibrium system set up by the enzyme, of the three acids citric represents 89.5%. Trans-aconitic acid, as Krebs and Eggleston [9] have shown, is easily converted to cis-aconitic.

The high content of aconitic and low content of citric acid in cereals, the absence of the former and large amount of the latter in legumes, is possibly the result of the different orientation of aconitase activity in these plants.

In legumes, as in cereals, seed germination – a transition from rest to vital activity – is associated with intense new formation of organic acids. However, in addition to the basic difference already noted, others are observed. In cereals during germination citric acid accumulation occurs in sprouts; here there is two or three times more than in the initial seeds. In kidney bean and bean, conversely, active consumption of the citric acid, contained in the seeds, is noted; only in later phases of leaf development does citric acid begin to accumulate.

Malic acid in both cereals and legumes, judging by the amount and tempo of formation, takes a very active part in metabolism. Accumulation of this acid proceeds far more actively in legumes during germination; in sprouts there is 27-53 times more than in the seeds producing them.

Leaves of cereals and legumes in organic acid content not only do not concede much to fruits and berries, distinguished by an acid taste, for example plum, cherry, currant, but even considerably exceed such fruits as apple, pear, tomato [10], and are therefore a convenient object for study of acid metabolism in higher plants.

SUMMARY

Seeds of legumes (kidney bean, bean and pea) contain 0.8-2.12% organic acids.

The organic acids of legumes are citric, malic, acetic and other acids of as yet unestablished nature. Citric and malic acids represent 45-85% of the total acid content.

In seeds and sprouts of kidney bean and bean, citric acid predominates, and in leaves of pea, malic acid.

In distinction to cereals, in legumes aconitic acid is absent. During germination of legume seeds there occurs intense new formation of malic acid and consumption of citric acid. Content of the former increases 27-53 times and the amount of citric acid is reduced to less than half.

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RIBONUCLEIC ACID OF PLASTIDS AND ITS CHANGES DURING PLANT DEVELOPMENT

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Study of the nucleic acids, their formation and changes, is of importance not only for an understanding of the mechanism of metabolic processes, but also of many other problems related to growth and organism development, in particular, to protein synthesis in the living cell. Of great interest in this respect is investigation of the nucleic acids of individual structural elements of the cell. In recent years considerable progress has been made in study of distribution of nucleic acids in the animal cell; it has been shown, for example, that of the protoplasmic structures, the richest in ribonucleic acid (RNA) are the microsomes.

But with respect to plants, literature data are lacking on systematic investigation of nucleic acids of the cell structures.

The idea of the possible presence of nucleic acids in chloroplasts was first expressed by Zaleski in 1911 [1]. Later (in 1938) Menke [2] isolated from defatted chloroplasts of spinach (by extraction with 0.01 N NaOH and precipitation with CCl_3COOH) a protein fraction, containing 0.7% phosphorus, and termed it nucleoprotein. Menke did not make a more detailed study of this fraction. In 1947 Euler and Hahn [3], studying nucleic acids of green leaves, found that part of the RNA, isolated by them from spinach leaves, occurs firmly bound to chlorophyll. In 1948 Euler et al. [4] isolated from spinach chloroplasts a protein preparation – chloroplastin. On the basis of the determination of pentoses by the phloroglucinol method the authors concluded that chloroplastin preparations contain 5-7% RNA. The amount of desoxyribonucleic acid (DNA), determined according to Dische, was very insignificant. Later Euler and Hahn [5] showed that the RNA values must be considered too high in view of the presence in plant material of pentose-containing compounds different from RNA and of pectin-like substances, also giving a positive reaction with phloroglucinol.

All these studies, however, have not provided a basis for a definite conclusion concerning the presence of nucleic acids in chloroplasts, and thus the question has remained unsettled.

In 1952 in our laboratory work was begun on determination of nucleic acids in various types of plastids [6]. In these studies, in addition to biochemical methods, the luminescent microscope was used, it being established that the main mass of nucleic acids present in plastids is RNA. Only on the basis of a very sensitive luminescent microscope method can the presence be detected in the plastids we studied of very insignificant amounts of DNA.

In the same year Metzner [7] published a paper on histochemical determination of nucleic acids in plastids of various plants. On the basis of his studies the author concluded that in the plastid stroma only RNA is present, but in the grana are both RNA and DNA. It should be noted that the stain used by Metzner can not be considered sufficiently specific for DNA.

In 1954 Jagendorf and Wildman [8] published a paper on the investigation of certain biochemical properties of isolated tobacco chloroplasts. The authors directed particular attention to the method of isolation of plastids. In order to isolate chloroplasts in intact form and without admixture of other submicroscopic structures, they were

repeatedly washed. Plastids so isolated contained 0.3-0.7% nucleic acids of the dry weight, about 2/3 of this being DNA. This paper called forth a number of criticisms of the methodology. Repeated washing, recommended by the authors, as was shown experimentally [9], can lead to considerable loss of nucleic acids. Furthermore, the prolonged period in the dark employed in the work can not fail to produce changes in chemical composition of the chloroplasts.

In the same year Straus [10] in a study of the chemical composition of chromoplasts found that the nucleic acid content reaches 0.72-1.03% of the dry weight. Thus, information in the literature on plastid nucleic acids is fragmentary and contradictory.

We conducted further studies of plastid ribonucleic acid [11] with consideration of the physiological state and ontogenetic development of the organism.

In the present paper the results are given of many experiments on chloroplasts from leaves of sugar beet, tobacco, spinach, clover, sunflower, and also on carrot chromoplasts and leucoplasts of roots of sugar beet. The studies have shown that the amount of RNA in plastids of various plant species varies and ranges from 0.5-3.5% of the absolutely dry weight. Furthermore, RNA content in chloroplasts of the same plant changes in the course of development. In certain cases the amount of RNA in chloroplasts from young leaves exceeds by approximately 3 times its content in those from old leaves. Thus, the amount of RNA in chloroplasts from leaves of the 4th rosette of sugar beet represents 0.6% with respect to phosphorus, and in those from leaves of the 12th rosette at the same period 1.7%.

A similar relation is observed for chloroplasts from leaves of tobacco and sunflower (Table 1).

TABLE 1

Change in Ribonucleic Acid Content in Chloroplasts of Tobacco "Trapezone" and Sunflower "Omsky skorospely" according to Tiers and Stages of Development.

Tier of leaves	Stage of development and sampling date	RNA content in % of air-dried wt. of plastids		Tier of leaves	Stage of development and sampling date	RNA content in % of air-dried wt. of plastids	
		from phosphorus	from purine			from phosphorus	from purine
Tobacco chloroplasts				Sunflower chloroplasts			
Upper	Budding	1.76	1.85	Upper	Budding	2.47	2.70
Middle	VII/27/1954	1.23	1.35	Middle	VII/19/1954	1.93	1.85
Lower		1.04	1.12	Lower		1.65	1.80
Upper	Flowering	0.89	0.97	Upper	Flowering	1.25	1.42
Middle	VII/29/1954	1.24	1.33	Middle	VIII/5/1954	1.35	1.50
Lower		1.18	1.30	Lower		1.39	1.57
Upper	Seed ripening	1.45	1.60	Upper	Seed ripening	1.49	1.45
Middle	VIII/24/1954	1.31	1.39	Middle	IX/6/1954	1.22	1.34
Lower		1.08	1.18	Lower		1.15	1.30

It is important to note that the usual character of the change in RNA content in chloroplasts of tobacco and sunflower in relation to change in physiological state of the plant is disturbed only in the flowering stage, when chloroplasts of young leaves become somewhat poorer in RNA than those of old leaves. This is apparently related to transport of RNA to flowers at this stage. Thus, while in the budding stage the RNA content in chloroplasts from tobacco leaves of the upper tier represents 1.76% with respect to phosphorus, and in those of the lower tier 1.04%, we have in the flowering stage 0.89 and 1.18%, respectively. In the seed ripening stage, as a rule, the earlier picture of RNA distribution was reestablished.

TABLE 2

Changes in Ribonucleic* Acid Content and Ribonuclease Activity of Chloroplasts during Plant Development.

Stage of development and sampling date	Preparation	RNA content in % of air-dried wt. of acetone preparations of plastids	Ribonuclease activity in mg P per 100 mg total N
		Sugar beet chloroplasts	
IX/17/1954	of 4th rosette	0.80	5.91
	of 12th rosette	2.10	2.14
		Sunflower chloroplasts	
IX/17/1954	Upper tier	1.50	1.18
Seed ripening	Lower tier	1.23	1.45
		Tobacco chloroplasts	
X/5/1954	Upper tier	3.72	1.27
Budding	Lower tier	1.63	2.40
X/5/1954	Upper tier	2.10	1.73
Flowering	Lower tier	2.77	1.55
X/5/1954	Upper tier	1.99	2.16
Seed ripening	Lower tier	1.67	2.35

From our experiments it follows that in chloroplasts, as in other biological material, fundamental changes are observed in RNA content in relation to physiological state and aging characteristics of the organism.

Together with study of change in RNA content of chloroplasts we also studied their ribocnuclease activity during plant development.

Determination of ribonuclease activity in most experiments was made according to the method of McFadden as modified by Chepinoga, Skvirskaya and others [12] with certain changes.

The reaction mixture in our experiments was incubated 1 hour in a thermostat at 25° (this temperature was chosen in order to approximate physiological conditions). Thymol was used as antiseptic during incubation. Ribonuclease activity was expressed in milligrams of unprecipitated phosphorus found in the experiment, calculated on 100 mg of total nitrogen of a water suspension of chloroplasts.

The data we obtained from study of ribonuclease activity in chloroplasts of tobacco, sunflower and sugar beet during plant development are given in Table 2.

From these data it follows that there is complete correspondence between change in ribonuclease activity and change in amount of substrate, i.e. RNA. In proportion to plant development the ribonuclease activity of chloroplasts increases, and the amount of RNA falls correspondingly. Thus, for example, in going from the 12th rosette of sugar beet leaves to the 4th the ribonuclease activity of chloroplasts increases from 2.14 to 5.91 mg P per 100 mg N, and the amount of RNA falls at this time from 2.10 to 0.80% of the air-dried weight of acetone preparations of plastids.

The same relation was also found in sunflower chloroplasts in going from the upper, younger tier of leaves to the lower, older tier. However, in view of the fact that the experiment was set up very late, at the end of the stage of seed ripening when even the upper leaves had begun to turn yellow, this relation was considerably less marked.

The same thing may be said concerning tobacco chloroplasts at this stage of development; in this case also the difference in RNA content in leaves of upper and lower tiers and in ribonuclease activity is relatively small, since yellowing and withering of all the leaves had already occurred.

* In this table in calculation of chloroplast RNA content, the substances, extracted by acetone, were disregarded.

TABLE 3

Forms of Phosphorus Compounds in Plastids of Various Plants.
In % of total phosphorus

Acetone preparations of plastids	Stage of development and sampling date	Acid-soluble phosphorus		Nucleic phosphorus	Lipoid phosphorus *	Phosphoprotein phosphorus
		Total	Inorganic			
Chloroplasts:						
of 12th rosette of sugar beet	X/7/1952	50.8	42.3	38.0	7.8	3.4
of upper tier of tobacco	Budding VIII/17/1953	37.0	34.5	45.0	10.6	6.8
of spinach	VII/2/1953	47.5	44.0	32.5	12.0	8.5
Chromoplasts of carrot	IX/29/1953	61.0	55.5	29.0	5.5	4.5
Leucoplasts of roots of sugar beet	XI/20/1953	47.0	41.0	26.4	22.6	4.0

TABLE 4

Changes in Phosphorus Compounds in Chloroplasts of Sugar Beet "Uladovka" in Course of Development.
In % of total phosphorus

Preparation	Sampling date	Acid-soluble phosphorus		Nucleic phosphorus
		Total	Inorganic	
Chloroplasts of rosettes:				
2nd	VII/27/1953	53.0	35.3	22.3
4th	VII/27/1953	44.2	39.5	44.2
4th	VIII/20/1953	56.0	52.2	23.7
7th	VIII/20/1953	48.0	46.0	40.0
4th	X/15/1953	71.2	58.5	11.0
7th	X/15/1953	64.5	50.0	15.0
12th	X/15/1953	59.0	46.0	30.0
4th	X/7/1952	73.0	70.8	11.8
12th	X/7/1952	50.8	42.3	38.0

It is of interest to note that the relation we observed between the amount of RNA and ribonuclease activity in chloroplasts was maintained also in the flowering period. At this time, as already noted, chloroplasts from leaves of the upper tier were always somewhat poorer in RNA than those from leaves of the lower tier, and ribonuclease activity was correspondingly greater in leaves of the upper tier (1.73 and 1.55 mg P per 100 mg N, respectively).

The uniformity of change in ribonuclease activity in the course of plant development noted above was checked and confirmed by us by determination of the activity of this enzyme by the manometric method.

Thus, one cause of the varying RNA content in plastids is change in their ribonuclease activity.

With the purpose of establishing the relation of RNA to other phosphorus compounds we determined the forms of phosphorus compounds in plastids.

Results of some of these experiments are given in Table 3.

* The relatively low values for lipoid phosphorus are apparently related to its loss during acetone treatment of plastids.

From Table 3 is seen that the plastid preparations we studied are characterized by a high content of acid-soluble, chiefly inorganic phosphorus compounds. Inorganic phosphorus content reaches 80-90% of the value for the total phosphorus of the acid-soluble fraction. Since in our experiments values for lipoid and phosphoprotein phosphorus were very insignificant, in study of the relationship of the forms of phosphorus compounds in plastids in the course of plant development we limited the determinations to the acid-soluble, inorganic and nucleic forms of phosphorus.

In Table 4 are given the data we obtained on change in phosphorus compounds in sugar beet chloroplasts in the course of development.

In comparing the data of Table 4 a very clear parallelism appears between increase in amount of RNA phosphorus and the simultaneous fall in acid-soluble phosphorus content. Thus, in the July 27, 1953 experiment, in going from the 2nd to the 4th rosette of sugar beet leaves, when the nucleic phosphorus content increases from 22.3 to 44.2%, the acid-soluble phosphorus content falls from 53.0 to 44.2%, respectively. In most experiments the inorganic phosphorus varies similarly to the total phosphorus of the acid-soluble fraction and in going from chloroplasts from young leaves to old ones it also increases.

The contrasting character of the change in nucleic, acid-soluble and inorganic phosphorus can be observed in chloroplasts not only in going from young leaves to old ones on the same plant, but also according to the degree of aging of leaves of a given rosette, that is, according to the stage of the vegetative period. Thus, for example, the nucleic phosphorus content in chloroplasts from leaves of the 4th rosette July 27, 1953 is 44.2%, and October 15, 1953 only 11% of the total phosphorus; acid-soluble phosphorus meanwhile increases from 44.2% to 71.2%, respectively, and inorganic phosphorus – from 39.5 to 58.5% of total plastid phosphorus.

We observed an analogous interrelation of the various forms of phosphorus compounds in chloroplasts of tobacco and sunflower depending on stage of plant development and position of the leaf tier (Table 5).

From Table 5 is seen that in chloroplasts of tobacco and sunflower also, as in sugar beet chloroplasts, to greater nucleic phosphorus content there corresponds lesser content of acid-soluble and inorganic phosphorus, and vice versa.

The high percent of acid-soluble phosphorus and especially the inorganic at the flowering stage is striking. At this period the acid-soluble phosphorus content in chloroplasts from all three tiers of tobacco leaves is about 60%, and of sunflower about 50%; • of the total plastid phosphorus. During flowering there evidently occurs an intensified mineralization of phosphorus related to its transport to flowers.

The analytical data we obtained on change in phosphorus compounds in chloroplasts of sugar beet, tobacco and sunflower in the course of plant development indicate that in plastids the phosphorus of the acid-soluble fraction, evidently the inorganic phosphorus, is the source of phosphorus for RNA biosynthesis.

Proceeding on the basis of recent literature data on the qualitative differences in nucleic acids, we investigated the qualitative composition of plastid RNA. We studied RNA from chloroplasts of sugar beet and tobacco, isolated from plastids in the course of plant development.

Batches of acetone preparations of plastids (1-2 g) were successively treated with cold 0.2% $HClO_4$ (twice) to remove acid-soluble compounds and with an alcohol-ether mixture (3:1) with boiling to completely remove lipoids after which RNA was extracted by treatment of plastids three times with 1 N $HClO_4$ in a thermostat at 37° each time for 12-15 hours. Then plastids were washed with cold 1 N $HClO_4$ and all the acid extracts combined. After this treatment the residual mass contained no phosphorus or purine base nitrogen. The combined extracts from plastids, containing RNA decomposition products of varying degree of complexity, were concentrated under vacuum on a sand bath with mild heating to the appearance of thick white vapors. Perchloric acid hereupon became 65-70% (d ~ 1.65). The concentrated RNA extract was transferred to an ampul and hydrolyzed to free bases in a thermostat for 2 hours at 100° [13]. The hydrolyzate was somewhat diluted with water, filtered from humus substances and neutralized with 40% KOH to weak acid reaction (pH~5-6). The $KClO_4$ precipitate was removed by centrifugation and carefully washed with water. Hydrolyzate and water washings were again concentrated under vacuum to a small volume, separated by centrifugation from the precipitated salts, and then taken to dryness in a vacuum desiccator. The dry residue was diluted with 1 ml distilled water

• Inorganic phosphorus in the former is about 55%, and in the latter about 50%.

TABLE 5

Changes in Phosphorus Compounds in Chloroplasts of Tobacco "Trapezond" and Sunflower "Omsky skorospely" according to Tiers and Stages of Development.
In % of total phosphorus.

Tier of leaves	Stage of development and sampling date	Acid-soluble phosphorus		Nucleic Phosphorus
		Total	Inorganic	
		Tobacco chloroplasts		
Upper	Budding	37.0	34.5	45.0
Middle	VIII/17/1953	45.0	43.0	41.0
Lower		50.0	42.5	30.5
Upper	Flowering	60.0	55.5	32.0
Middle	IX/1/1953	57.0	54.5	25.0
Lower		60.0	55.5	30.0
Upper	Seed ripening •	34.0	31.0	50.0
Middle	IX/30/1953	52.0	50.5	35.5
		Sunflower chloroplasts		
Upper	Budding	34.7	28.2	52.1
Middle	VII/19/1954	36.8	29.0	50.0
Lower		46.0	35.1	43.2
Upper	Flowering	47.9	44.8	41.3
Middle	VIII/5/1954	49.3	43.7	42.6
Lower		50.3	47.0	40.0
Upper	Seed ripening	35.1	30.0	38.9
Middle	IX/6/1954	39.6	33.0	40.0
Lower		56.3	44.2	30.3

and kept in the icebox under toluol. Definite amounts of the hydrolyzate so obtained were applied with a micropipet to paper and subjected to chromatographic analysis for 2-3 days in a mixture of 6 volumes isopropyl alcohol, 2 volumes 95% ethyl alcohol and HCl, added to pH – 1.00 of the whole solution.

The position of the RNA nitrogen bases on the chromatogram was detected in the ultrachemiscope in the form of dark spots on a fluorescing background. Then the spots were cut out and the bases eluted with 5 ml 0.1 N HCl at room temperature for 15-20 hours, after which the absorption spectrum of each eluate was taken in ultraviolet light. From extinction values at maximum absorption and the corresponding molar extinction coefficients the nitrogen base content of RNA was calculated.

Our experiments on the qualitative composition of plastid RNA showed that its proportions of nitrogen bases are not equimolecular. In all RNA samples studied the purine bases predominated over the pyrimidine, and the amount of guanine and cytosine in a number of cases exceeded the amount of adenine and uracil. We did not succeed in finding definite regularities in change in content of individual nitrogen bases of RNA in the course of development. We are not presenting detailed data on the qualitative composition of plastid RNA, since an evaluation of such data must be approached with great care. In the present state of methods for quantitative chromatography of nucleic acids, for their extraction from tissues and especially of methods for hydrolysis of these compounds the possibility of a number of methodological errors is not excluded.

Furthermore, the composition of RNA in our experiments may have been to a certain extent changed owing to ribonuclease action during isolation of chloroplasts. We did not inactivate it in the process of isolation, since

• At this stage leaves of the lower tier had yellowed and fallen.

according to data of Granick [14] any added salts even in insignificant concentrations have a tendency to precipitate the cytoplasmic proteins, and then it is very difficult to wash plastids free from the adherent cytoplasm. As a matter of fact, according to recent reports in the literature [15], plant ribonuclease splits all the bonds of RNA, in distinction to pancreatic ribonuclease, which splits primarily the bonds between pyrimidine nucleotides. But it has not yet been experimentally demonstrated sufficiently convincingly, whether plant ribonuclease splits all RNA bonds at the same rate.

In studying chloroplast RNA we found that RNA is bound to a protein complex of the plastids; a certain part of it being bound more loosely is easily extracted by 0.14 M NaCl; the other part is bound more firmly and can be isolated only after treatment of plastids with 0.2% NaOH or stronger agents. The degree of firmness of the RNA bond with the protein complex of plastids does not remain constant during the course of plant development. From chloroplasts of young leaves practically all the RNA is extracted by the action first of 0.14 M NaCl and then 10% NaCl with boiling, while in chloroplasts of old leaves after such treatment there still remains a significant amount of RNA, which is extracted by 0.2% NaOH. In proportion to development there seems to occur a fixing of RNA in the structures. It is possible that thus an "exclusion" of RNA from the dynamics of metabolism is attained.

SUMMARY

Chloroplasts from leaves of various plant species contain 0.5-3.5% RNA on an absolutely dry weight basis, depending on the nature, age and physiological state of the plant.

RNA content of chloroplasts undergoes uniform changes in the course of plant development; chloroplasts of young leaves contain about 2-3 times more RNA than those from old leaves.

The usual type of RNA distribution in chloroplasts is disturbed at the flowering stage, which is probably due to its transport to flowers at this period.

Simultaneously with decrease in amount of RNA in chloroplasts in the course of plant development there is observed a rise in their ribonuclease activity.

Together with the considerable increase in nucleic phosphorus there occurs a reduction of acid-soluble phosphorus. Apparently the latter is the source of phosphorus in plastids for RNA biosynthesis.

RNA is not all bound in the same way to the protein complex of plastids. Part is bound more loosely and is extracted by weak solvents, such as 0.14 M NaCl; the other part is bound more firmly and for its extraction stronger solvents are necessary (for instance, 0.2% NaOH).

In proportion to plant development together with general decrease in amount of RNA in chloroplasts there seems to occur a fixing of it in the structures, and the amount of firmly bound RNA is increased.

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QUANTITATIVE DETERMINATION OF POTATO GLYCOALKALOIDS AND THEIR PREPARATIVE SEPARATION

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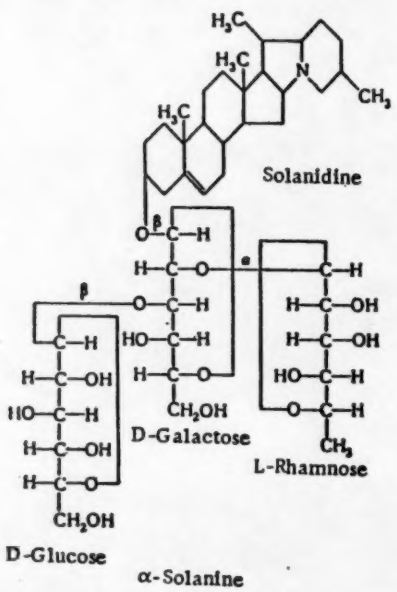
Until recently it had been considered that the cultivated form of potato contains only one alkaloid, solanine, discovered 135 years ago. Substances of the glycoalkaloid type have attracted special interest of workers in the last decade in connection with the discovery of the toxic action of some of them on the Colorado beetle.

In 1954 Kuhn and Low [1] reported the discovery in leaves and shoots of cultivated potato of another glycoalkaloid, α-chaconine. They also found chaconine in leaves of the wild potato Solanum chacoense, from which it was named.

Chaconine and solanine have the same aglucone – solanidine, but differ with respect to the sugar chain. The sugar portion of α-solanine is a trisaccharide, composed of galactose, glucose and rhamnose. According to recent data [1, 2], the trisaccharide of α-solanine, termed solatriose, has a branching structure.



α-Solanine

The sugar portion of α-chaconine is also a trisaccharide, but is composed of 1 molecule of glucose and 2 molecules of rhamnose. The order of distribution of the sugars is as follows: solanidine – glucose – rhamnose – rhamnose. Besides these main alkaloids, representing up to 95% of the total alkaloids, in leaves of S. tuberosum and S. chacoense were found four more glycoalkaloids, termed β- and γ-solanine and β- and γ-chaconine. They have a shortened sugar chain, in the β-forms a disaccharide, and in the γ-forms a monosaccharide:

α-solanine: solanidine – galactose – glucose – rhamnose
β-solanine: solanidine – galactose – glucose
γ-solanine: solanidine – galactose
α-chaconine: solanidine – glucose – rhamnose – rhamnose
β-chaconine: solanidine – glucose – rhamnose
γ-chaconine: solanidine – glucose

Discovery of these new glycoalkaloids was made possible by use of paper chromatography and column chromatography. Separation and preparative isolation of these alkaloids was accomplished by Kuhn and Low

on a column of aluminum oxide [3, 4]. These authors used paper chromatography for qualitative determination and control of purity of fractions from the column.

For study of the pathways of glycoalkaloid biosynthesis and physiological role in metabolism of the potato plant, it was necessary to have a method for their quantitative individual determination.

The previously used method [5] permitted determination only of total glycoalkaloids in potato.

Separation of glycoalkaloids on an Al_2O_3 column, employed by Kuhn, does not assure clear demarcation of one glycoalkaloid from another and can not therefore be used for quantitative determination in large-scale analyses. Attempts to utilize Kuhn's data for chromatographic separation of glycoalkaloids on domestic brands of paper were not successful. With the solvent ethyl acetate – acetic acid – water, 3:1:3 (the upper phase was used, to which was added 15% ethanol) α-solanine and α-chaconine were not separated.

We obtained clear separation of solanine and chaconine by chromatography on paper, saturated with M/5 monopotassium phosphate solution, and use as solvent of the mixture: ethyl acetate – acetic acid – water, 11:2:2.

Munier and Macheboeuf [6] pointed out the advisability of saturating paper with buffer salts in chromatographing alkaloids of the tropane group and certain other alkaloids. The excellent separation of glycoalkaloids on saturated paper made possible a method for their quantitative individual determination.

Quantitative Determination by Paper Chromatography of Solanine and Chaconine of Potato

Absolutely dry material, ground and sifted, was taken for analysis. Since content of solanine and chaconine is not the same in various organs of the potato plant, it is advisable to use the following amounts: for tubers 50-60 g, for leaves 10-20 g, for shoots 0.75-1.0 g. A batch of material was diluted with 50-100 ml 2% acetic acid and shaken in a tightly closed flask on a shaker for 2 hours. Then contents of the flask were filtered in a Büchner funnel and the material again extracted in the same way. Filtrates were combined, made alkaline with concentrated ammonia to phenolphthalein and heated to 70-80°. The precipitate was collected on a paper filter and washed 2-3 times with water made slightly alkaline with ammonia. The filter with precipitate was dried and treated on the glass filter with boiling ethyl alcohol (in the amount of 200-250 ml). The alcoholic solution of glycoalkaloids was placed in a Wurtz flask, and the alcohol evaporated on a water bath to dryness. The residue in the flask was dissolved in an amount of 2% acetic acid, such that the final concentration of glycoalkaloid in the solution was 0.5-1%. Our experiments showed that it is necessary to apply in one spot from 100 to 250 μg glycoalkaloid (larger quantities are poorly separated). Therefore, before chromatographing, the alkaloid concentration must be determined in the solution obtained. For this, a part of the original solution was diluted 10-20 times and the amount of glycoalkaloid determined according to Alberti's reaction [4].

The chromatographic paper ("Leningrad fast") was first saturated with M/5 KH_2PO_4 solution and dried in a drying cupboard at 130°. To a sheet of paper, 13 × 30 cm, were applied 5 spots of a single preparation, 100-200 μg per spot. The amount of solution applied in one spot is calculated exactly and should be approximately identical.

The solvent was a mixture of ethyl acetate, acetic acid and water, 11:2:2. This ratio was used in order to avoid separation of the mixture into phases and thus to obtain greater constancy in the ratio of the parts.

Special attention must be given to the quality of the ethyl acetate. The latter must be absolutely pure, distilled over P_2O_5 (b.p. 77°). It should be kept in the refrigerator.

After application of glycoalkaloids the chromatograms were kept in a vessel, saturated with solvent vapors, for 1-1.5 hours, and only afterwards was solvent poured in. Chromatography – descending, for 14-15 hours, was based on running through of solvent. After separation the chromatograms were dried in air 1-2 hours, then along the edges of the chromatogram control strips 3-3.5 cm in width were cut off, on which were distributed the outer spots of the alkaloids. These strips were sprayed with 23% solution of $SbCl_3$ in chloroform and heated 5 minutes at 90° in a drying cupboard. The alkaloid spots become a raspberry color, but the color is not stable; after 3-4 days it turns gray, and the paper itself rapidly decomposes. The strips were placed next to the undeveloped middle part of the chromatogram, on which the distribution of spots was established according to the level of spots on the control strips. The section of paper corresponding to one spot was cut out in the form of a rectangle, very small pieces were cut, placed in a test tube, a small volume of 2% acetic acid added (2-3 ml) and left for 24

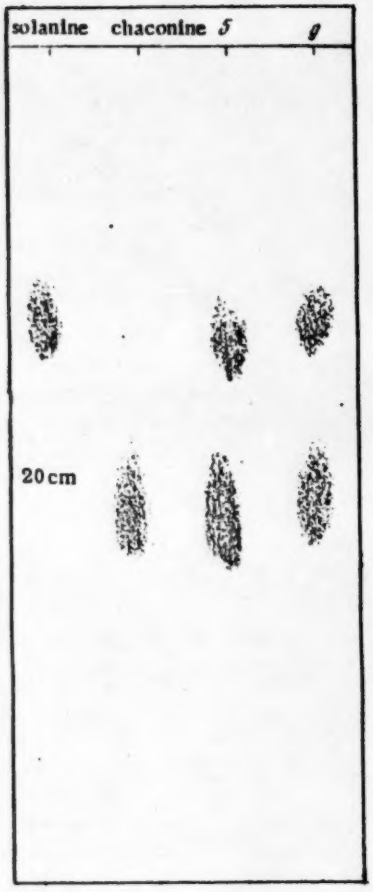


Fig. 1. Chromatogram of glycoalkaloids from shoots (♂) and leaves (♀) of potato.

TABLE 1

Determination of Solanine and Chaconine.

Substance	Applied in μg	Determined in μg	% recovery
Solanine	120	114	95
Solanine	104	100	96
Solanine	160	154	96
Chaconine	82	82	100
Chaconine	131	130	99
Solanine + chaconine	120+82	120+80	100;97
Solanine + chaconine	60+49	60+48	100;98

TABLE 2

Glycoalkaloid Content in Shoots and Leaves of Potato "Berlikhingen".
In % of absolutely dry wt.

Material	Total glycoalkaloids	Solanine	Chaconine
Shoots	5.3	1.96	3.24
Leaves	0.16	0.05	0.11

hours. During this time the tube was shaken several times. Then 1 ml of eluate was placed in a 50-ml Erlenmeyer flask, to which was added with cooling by drops from a burette 2 ml concentrated H_2SO_4 + 1 ml 1% formaldehyde solution (Alberti's reaction). A rose color develops within 45-60 minutes. Color measurements were made in a photoelectric colorimeter with a blue light filter. Readings were compared with a calibration curve and the amount of glycoalkaloid in the spot was found. Measurements were made in triplicate; from the average value the ratio of the glycoalkaloids was calculated. Since the color is produced by the by the aglucone, α-solanine and α-chaconine can be determined from the same calibration curve.

In order to check the accuracy of the method and its suitability for determination of solanine and chaconine in mixtures, to the chromatogram was applied an exact amount of each glycoalkaloid, and also a mixture, and they were determined by the method described above.

Table 1 shows that the accuracy obtained in the individual determination of glycoalkaloids permits the use of this method for analytical purposes.

Several organs of the potato plant were analyzed by this method.

In Table 2 is given the glycoalkaloid content of shoots and leaves of the potato variety "Berlikhingen". As markers on the chromatograms were used solanine and chaconine isolated by us from potato shoots (see Figure 1). R_f of solanine and chaconine was usually not determined, since these alkaloids move very slowly on the saturated paper, while the solvent moves very fast (6-7 cm per hour). Therefore the chromatograms were set up for running

through of solvent. For the distribution characteristic of spots relative to one another the coefficient $R_{\alpha s}$ was used: $R_{\alpha s} = \frac{\text{rate of movement of chaconine on paper}}{\text{rate of movement of solanine on paper}} = 2$

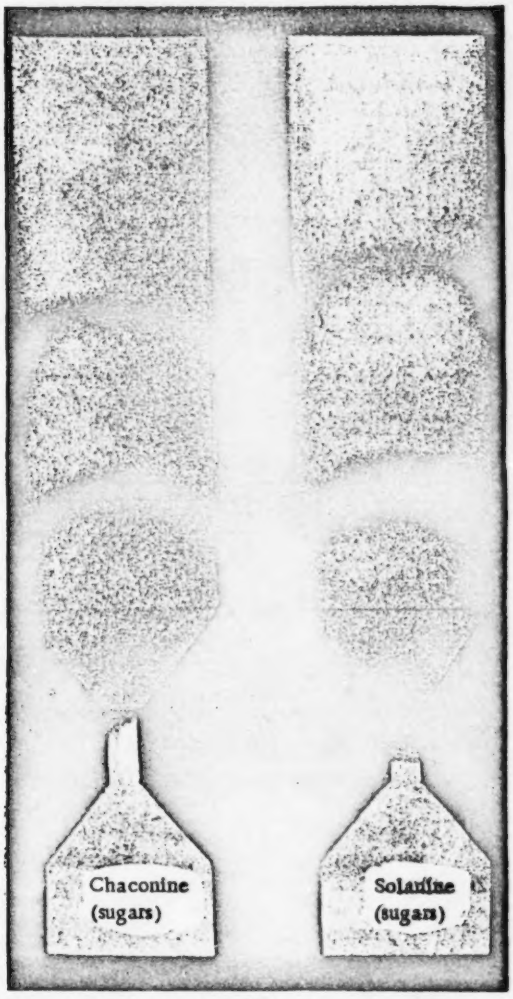


Fig. 2. Chromatograms of sugars from hydrolyzates of chaconine (sugars from below upward: glucose, rhamnose) and of solanine (sugars from below upward: galactose, glucose, rhamnose).

Separation of Glycoalkaloid Sugars on Paper

For determination of the composition of sugars in the glycoalkaloids solanine and chaconine, ascending paper chromatography was used; solvent: benzene, butanol, pyridine, water [7]. Chromatograms were cut in the form of strips with narrow necks [8] (Figure 2), to which was applied the alkaloid hydrolyzate. The solvent

TABLE 3

Alkaloid Distribution According to Column Fractions.

Fractions	Amount ml	Wt. of residue, mg	Glycoalkaloid composition
1	100	–	Contained no glycoalkaloid
2 – 4	100	330	Chaconine
5	30	35	Chaconine and traces of solanine
6	20	30	Chaconine and solanine
7 – 9	325	270	Solanine
	Total:	665	

combination indicated above and special shape of chromatograms permitted clear separation of galactose and glucose in mixtures. 20 mg glycoalkaloid (solanine or chaconine) was hydrolyzed 3 hours with 2 ml 1 N H_2SO_4 on a boiling-water bath. The hydrolyzate was neutralized with 10% NaOH to neutral reaction with universal indicator. The aglucone precipitate was filtered off and to the chromatogram was applied 20-30 μl of filtrate. Solvent was passed through twice – 10-12 hours each time. Chromatograms were dried in air and developed with aniline-phthalate reagent. The chromatograms obtained were compared with the control, to which was applied 10 μl of a mixture of 1% solutions of glucose, galactose and rhamnose.

From Figure 2 is seen that while in α-solanine 3 sugars are clearly visible – galactose, glucose and rhamnose, in α-chaconine only glucose and rhamnose are found qualitatively.

Separation of Glycoalkaloid Sugars on a Column

For preparation of pure chaconine, chromatography on a column of Al_2O_3 was used. A mixture of glycoalkaloids, obtained in the usual way from potato shoots, was used for separation. It was first established by paper chromatography that the mixture consisted of 40% solanine and 60% chaconine.

For separation of 0.65 g of the mixture, dissolved in 40 ml n-butanol saturated with water, a column 3.6 x 10 cm was used. The column was packed with dry "aluminum oxide for chromatography". For development of solanine n-butanol saturated with water was used. The eluate from the column was collected in separate fractions, whose composition was determined by paper chromatography. Fractions of the same composition were combined, n-butanol was evaporated under vacuum to dryness and the residue weighed.

From Table 3 is seen that in chromatographic separation on a column we obtained complete recovery of the amount of alkaloids applied. The amount of solanine and chaconine, obtained by separation on a column, agreed well with the data obtained on analysis of the preparation by quantitative paper chromatography. Consequently, the accuracy and suitability of the method of quantitative paper chromatographic determination of solanine and chaconine has been confirmed by the method of column chromatography. Chaconine after evaporation of butanol was dissolved in 96% hot ethyl alcohol. On cooling it crystallized in long, very fine needles, in the form of rosettes. After recrystallization from 80% alcohol chaconine melted with decomposition at 230-236°. Its optical rotation was: $[\alpha]_D^{20}$ = = – 85° (in pyridine).

The preparation of chaconine obtained upon chromatography showed complete homogeneity.

SUMMARY

Quantitative determination of potato glycoalkaloids by the method of paper chromatography is proposed.

Quantitative preparative separation of the glycoalkaloids was carried out on a column of aluminum oxide.

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ELECTROPHORETIC STUDY OF THE FRACTIONAL COMPOSITION OF SKELETAL MUSCLE PROTEINS OF VERTEBRATES IN ONTOGENESIS

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The capacity for contractility of muscle fibers is due to the presence of proteins specialized for this function.

The relation between change in physical state of these proteins and splitting of ATP was established by work of Engelgardt and Lyubimova [1] and by the Hungarian school of biochemists [2]. Together with proteins of the actomyosin complex, present in the somatic striated musculature of vertebrates, in the tonic smooth muscles of mesenchymal origin, performing a constrictor function and not capable of rapid contractile reactions, the presence was established by electrophoresis of a protein of somewhat different character (Ivanov and Blokhina [3]; see also [4, 5]). It should be emphasized that in the smooth motor musculature of invertebrates, having the capacity for contractile reactions of a tetanic character and being essentially a modification of musculature of the somatic type, proteins of the actomyosin complex are present in approximately the same amounts in which they occur in the striated musculature.

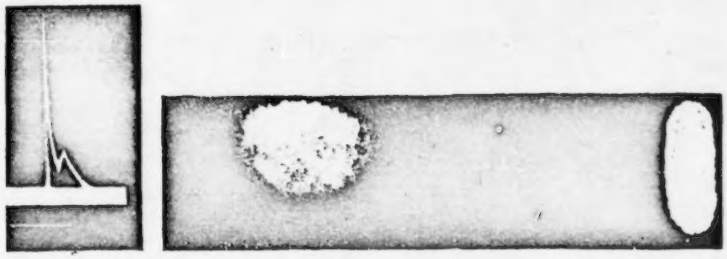
Ivanov and Kasavina [6], studying contractile proteins of the somatic muscles in ontogenesis, showed that in muscles of newborn rats and mice and in musculature of guinea pig embryos, proteins of the actomyosin complex can be found in but slight amounts. These data are in good agreement with the known fact that in the process of development of the nerve-muscle apparatus in embryonic and postnatal periods of life of the animal the tonic contractile reaction in course of time is replaced by the nontonic (Orbeli [7]).

In the present paper the results are given of an electrophoretic study of the fractional composition of the somatic musculature of animals (rabbits) at various stages of embryonic and postnatal development. Parallel with electrophoretic study of muscle extracts, observations were also made on the character of the contractile reaction of individual muscle fibers and protein threads in the presence of ATP.

METHODS

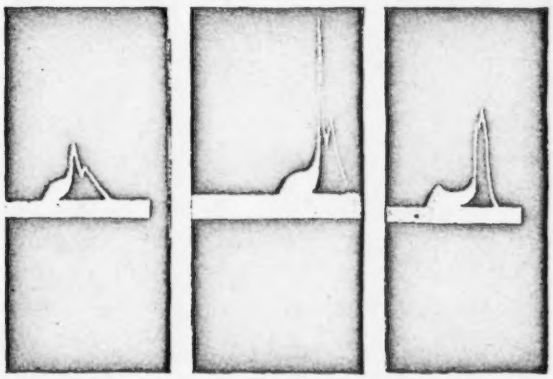
The striated musculature of rabbits was studied (dorsal muscle, muscles of the extremities). Adult animals were killed by air embolus, newborn and embryos – by decapitation. Skinned carcasses of animals were kept on ice until used. Excised muscles were lightly washed with cold twice-distilled water to remove blood and then ground in a cold mortar with quartz sand. Further treatment of the homogenate and preparation of a protein extract suitable for electrophoresis was carried out by methods described previously [3]. The slightly opalescent protein solution was dialyzed in the cold with stirring against phosphate buffer (0.048 M Na_2HPO_4, 0.006 M NaH_2PO_4 and 0.25 M NaCl) of ionic strength 0.4 and pH 7.8. On completion of dialysis the protein solution was diluted with buffer solution to 0.7-0.8%.

Electrophoresis was conducted in an apparatus of the Tiselius-Svenson type for 4-4.5 hours at 75-80 v and current strength 20 ma.



a b

Fig. 1. Electrophoregrams of striated muscle proteins of adult rabbit: a – free electrophoresis; b – paper electrophoresis.



a b c

Fig. 2. Electrophoregrams of striated muscle proteins: a – 3-day-old rabbit; b – newborn rabbit; c – rabbit embryo.

The relative percent content of individual fractions was calculated by measurement of the ratio of the areas under individual peaks.

Parallel with electrophoresis according to Tiselius analyses were made by paper electrophoresis of the same protein extracts. Electrophoresis was carried out on "fast" chromatographic paper (Volodarsky plant) at potential gradient 4-6 v/cm and current strength 0.8 ma per cm cross-section of the paper strip (paper strips 30 × 3 cm, distance between electrode sections about 20 cm). Protein solution was applied to the paper in the amount of 0.02-0.04 ml in the form of a streak perpendicular to the long axis of the paper strip at the edge immersed in the solution of the cathode section. Phosphate buffers of ionic strength 0.2-0.4 and pH 7.8 were used. Electrophoresis was continued 8-12 hours at 4°. Part of the experiments were conducted with entirely satisfactory results at room temperature. The dry electrophoregrams were heated in a drying cupboard at 105° for 5 minutes, then colored in the usual way with bromphenol blue. Determination of the color density of various sections of the paper electrophoregrams was made by means of a densitometer. For determination of the relative content of individual fractions, curves were constructed from the data: optical density – length of the respective section of the electrophoregram and then areas under the individual peaks were measured.

Fig. 3. Electrophoregram of striated muscle proteins of rabbit embryo.

RESULTS

By means of Tiselius electrophoresis electrophoregrams were obtained of striated muscle proteins of the adult rabbit (2 cases), 3-day-old rabbit (2 cases), newborn (1 case) and embryos of 24-26 days of fetal life, i.e. killed approximately 4-6 days before normal delivery (2 cases). Considerably more cases were studied by paper electrophoresis. It must be noted that on the electrophoregrams no significant individual differences were found in composition of muscle proteins of animals of the same age.

In Figure 1 are given the electrophoregrams we obtained of muscle proteins of the adult rabbit. In Tiselius electrophoresis (Figure 1, a) two peaks are clearly shown: I corresponding to the "actomyosin" and II – to the "myogen" fractions.* This picture is very well known from the literature.

In paper electrophoresis likewise two spots are clearly revealed, due to the presence of two protein fractions (Figure 1, b).

The fractional composition of muscle proteins of newborn animals (Figure 2, a and b) and embryos (Figure 2, c) differs considerably from that of muscle proteins of the adult animal.

Attention is first attracted to the appearance on these electrophoregrams of a new more mobile fraction. Facts known in the literature concerning the occurrence of anomalous peaks during protein electrophoresis, due to the presence at the boundary protein – buffer of a salt concentration gradient, compelled us to approach very carefully the evaluation of the electrophoretic picture obtained. Usually anomalous peaks arise in electrophoresis of concentrated protein solutions close to the position of the initial boundary. In our experiments protein concentration was relatively low, and the new fraction had the greatest mobility. However, Moore and Lynn [8] observed in electrophoresis of blood plasma proteins the appearance of a peak of greater mobility than albumin, but which was found to be anomalous.

Possible doubt as to the true protein nature of this new electrophoretic fraction is excluded by the results of repeated experiments, in which definite changes in it were observed, depending on the object of study. The area, formed by the new peak, and consequently the relative concentration of the protein fraction corresponding to this peak, increased uniformly with decrease in age of animals from whose muscles the extract was obtained.

The data on paper electrophoresis of muscle extracts of newborn animals were also very graphic. In this case there were on the electrophoregram instead of two – three protein spots (Figure 3).

Together with appearance of a new peak on the electrophoregram of protein extracts of muscles of newborn animals and embryos there are also observed other changes. Parallel with increase in area of the new peak, the peak corresponding to the "actomyosin" fraction decreases and almost vanishes.

In the table the percent content of protein fractions in muscle extracts of the adult and newborn rabbit and embryo is given from the electrophoretic data.

There is observed a clear inverse relation between percent content of proteins of fraction I and of the "actomyosin" fraction, while percent content of the "myogen" fraction changes little.

* Here, as later, the serial numbering of peaks is given from left to right, while protein migration proceeds from right to left.

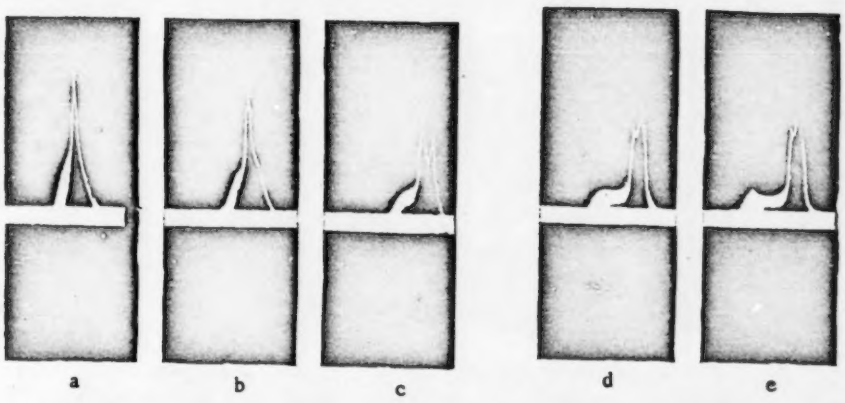


Fig. 4. Electrophoregrams of striated muscle proteins of rabbit embryo at single successive stages of separation:
a – e – single successive stages of electrophoresis (see text).

Content of Protein Fractions in Muscle Extracts of Rabbits

Rabbits	Content of protein fractions in %		
	I	II	III
Adult	0	56.7	43.3
Newborn	21.8	35.9	42.3
Embryo	34.5	12.7	52.8

In connection with what has been said it was of great interest to follow the course of electrophoretic separation of muscle proteins of embryos with time. In Figure 4 are shown separate stages of this process. In Figure 4, a, b and c is seen the gradual stratification of the protein with formation of three peaks. Later peaks I and III become clearer and the areas under them increase. In the latter two stages of electrophoresis (Figure 4, d and e) it is seen that peak II, which earlier (Figure 4, c) might have been taken for "actomyosin", gradually stratifies, and the area under the first peak simultaneously increases. The form of peak I – thickened contour, diffuse boundary – indicates nonhomogeneity of the respective protein fraction.

It should be noted that to the difference in fractional composition of the muscle extracts there also correspond other features of these proteins. Muscle fibers of newborn rabbits, which have been water-soaked, in agreement with data published previously [6], have less contractile capacity on addition of ATP than water-soaked fibers of adult animals. Water-soaked fibers of embryo muscles in general do not display this capacity. Protein threads contract to a lesser degree in the presence of ATP, the younger the animal from whose muscles they were obtained. Threads, obtained from muscle proteins of embryos, displayed minimal capacity to contract in the presence of ATP.

SUMMARY

In the embryonic and early postnatal period of animal development profound changes occur in fractional composition of proteins of the striated musculature in the direction of gradual enrichment in the "actomyosin" fraction, whose precursors evidently are proteins of the "proactomyosin complex".

These shifts in fractional composition of proteins of the striated musculature in ontogenesis are clearly shown in electrophoretic study of muscle proteins, dissolved in salt media of ionic strength about 0.4. It is entirely possible that similar shifts also occur during ontogenesis in composition of proteins of the muscle stroma, which are not extracted with Weber's solution. This problem is undergoing further study.

With respect to content of proteins of the actomyosin complex, embryonic muscles approach the smooth tonic muscles of mesenchymal origin of vertebrates, which also corresponds to their physiological type of contractile reaction.

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THE INTERACTION BETWEEN BORATE AND DEXTRAN

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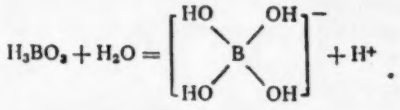
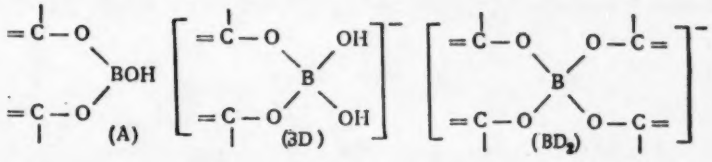
The ability of borate to react with polyhydroxyl alcohols has been repeatedly utilized for both preparative and analytical purposes. Borate reacts especially actively with those polyhydroxyl alcohols, whose adjacent OH-groups are in the cis position. The borate-diol complex formed manifests strong acidity and a number of other physicochemical properties, distinguishing it from the original diol.

Boric acid dissociates in water according to the reaction:



Depending on the ratio of concentrations of borate and polyhydroxyl substance, complexes can be formed of the types:



Compounds of the BD_2 type arise when there exists a considerable excess of hydroxyl groups. Gradually, with increase in borate concentration the amount of complexes of the BD type increases. In concentrated borate solutions the amount of compounds of type A increases [1].

The reaction of borate with polysaccharides proceeds essentially analogously to its reaction with simple substances, for example with monosaccharides, although here some differences are observed. In the first place it should be pointed out that acidity of the solutions does not undergo great change, in spite of the fact that with formation of strongly electronegative complexes of the BD and BD_2 type this would be expected. Characteristic features of polysaccharide-borate complexes are also their increased viscosity, molecular weight and electrophoretic mobility [2-5].

These facts are readily understandable with respect to polysaccharides, containing in their molecule simple carbohydrates with free adjacent hydroxyl groups in the cis position, as for example galactose and mannose. Polysaccharides of this kind (polygalactans, polymannans, blood group substances) were the object of a study of the reaction mechanism of borate with polysaccharides. It was found, however, that polysaccharides, composed exclusively of carbohydrate, having no cis-hydroxyl groups – of glucose, also display in borate buffers an increased electrophoretic mobility [6].

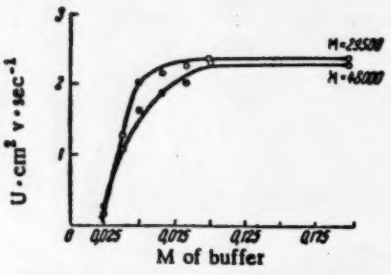


Fig. 1. Relation of electrophoretic mobility of dextran to molarity of borate buffer.

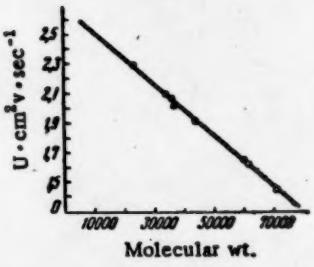


Fig. 2. Relation of electrophoretic mobility of dextran to its molecular weight.

In connection with what has been said it was of interest to determine whether dextran (1, 6-poly-α-glucosan) displays electrophoretic mobility in borate solution. The experiments described in the present paper have shown that this actually occurs and that the rate of electrophoretic migration is directly proportional to molecular weight of the polysaccharide (within the limits 20,000-60,000).

Precise determination of the structure of the dextrans used in the experiments made it possible to further reveal the mechanism of the interaction of borate with polyglucosan. and led to the conclusion that the complex formed is an acyclic borate derivative, that is, in a state of complete saturation each hydroxyl group of glucose is substituted with one boric acid radical.

EXPERIMENTAL

Material and Methods

Dextran. Dextran fractions used for experiment were described previously [7, 8]. These fractions were obtained by addition of ethanol to clinical dextran – "polyglucan" [9] – with collection of precipitates at 40, 50 and 70% ethanol concentration. The isolated precipitates were dissolved, dialyzed and again precipitated at the respective ethanol concentration, this process being repeated two or three times. The final preparations were dialyzed against distilled water and lyophilized. Molecular weights of preparations so obtained were calculated by the formula:

$$M = \frac{4\pi}{3\bar{V}N^2}\left\{\frac{RT}{6\pi\eta D}\cdot\frac{f_0}{f}\right\}^3, \qquad (1)$$

where R – universal gas constant, T = absolute temperature, $\bar{V}$ – partial molar volume, η – viscosity of the medium in poises, D = diffusion constant, f_0/f – friction coefficient.

The asymmetry value was calculated from data on viscosity measurement by means of Heppler's viscometer, the diffusion constant was determined in a vessel of the Tiselius type (see [7]).

Borate buffer. Electrophoretic measurements were made in boric acid solutions, neutralized to pH 10. Their molarity is given in experiment protocols. pH was measured potentiometrically, with use of the glass ("alkaline") electrode.

Electrophoresis was conducted in a standard Tiselius cell 84 × 25 × 3 mm, at 2.5° in white light and current strength 20 ma. The various lyophilized preparations were dissolved in the respective buffer and dialyzed not less than 24 hours against the same buffer. Areas of individual peaks were calculated planimetrically [4]. Electrophoretic mobility was calculated separately from the descending and ascending boundaries, in the latter case correcting for the ε anomaly [5].

TABLE 1

Electrophoretic Mobility of Different Dextran Fractions.
In $cm^2 v^{-1} sec^{-1} \times 10^{-5}$

Boundary	Molecular weight							
	58 000		49 000	36 000	30 000		29 500	19 300
Ascending	1.38	1.46	1.67	1.93	1.98	2.03	1.99	2.27
Descending	1.46	1.45	1.63	1.90	2.05	2.07	2.14	2.33
Average	1.44		1.65	1.91	2.02		2.07	2.30

RESULTS

1. Relation between molarity of borate buffer and rate of dextran movement.

To determine the effect of molarity of borate buffer on rate of electrophoretic movement, dextran fractions of molecular weights 49,000 and 29,500 were used. These fractions were dissolved in 0.025-0.2 M boric acid solutions, brought to pH 10 with sodium hydroxide. Electrophoretic mobility data are given in Figure 1; circles on the graph correspond to an average of the values for the ascending and descending boundaries. Figure 1 shows that at high and at low borate concentrations the electrophoretic mobilities of dextrans of different molecular weights differ little from one another. However, in the middle region, from 0.045 to 0.085 M boric acid, the rate of movement of the heavy fraction is markedly less than that of the light fraction.

2. Relation of molecular weight to rate of electrophoretic movement.

In Table 1 are given the experimental data obtained for various fractions dissolved in 0.05 M borate buffer at pH 10.

By taking the molecular weight values of the various fractions and the corresponding averages of the results in Figure 1, the relation can be shown graphically between molecular weight and rate of movement (Figure 2). The straight line in Figure 2 was plotted according to the method of least squares and is expressed by the equation:

$$u = 0.21 \cdot 10^{-4} M + 2.69.$$

3. Separation of dextran mixtures.

The linear relation shown above between molecular weight and electrophoretic movement indicated the possibility of an electrophoretic separation of a mixture of different dextran fractions in ~ 0.5 M borate buffer. To check this assumption two-component mixtures of dextran fractions in 0.05 M borate buffer, pH 10, were used. Solutions of the fractions were dialyzed against the same buffer and studied electrophoretically. The curves obtained are given in Figure 3 (a-d), results of calculations and composition – in Table 2.

Table 2 shows that for mixtures, containing fractions with relatively large differences in molecular weight (18,000-58,000), the quantitative recovery is very good (about ± 7%). The electrophoretic mobility of each component of the mixtures corresponds (within limits ±10%) to that of the given component, determined separately. When the differences in molecular weight are reduced, accuracy is reduced of determination of the degree of participation of each component in the mixture, and separation in the electric field can not be attained, at least in the course of the running time we usually employed (about 20,000 seconds).

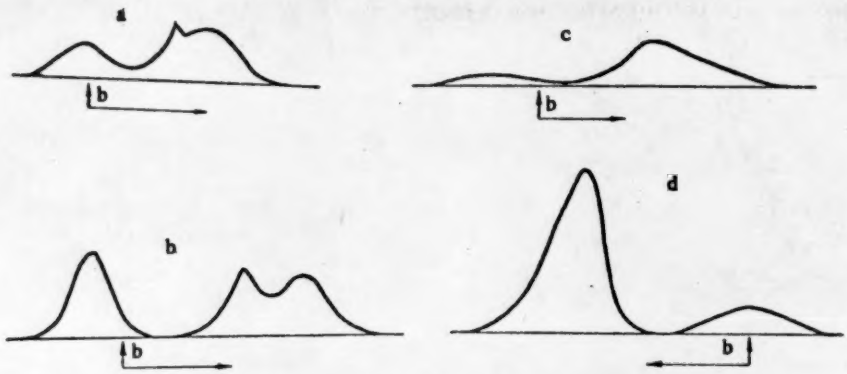


Fig. 3. Electrophoretic curves of mixtures of dextran fractions of different molecular weights:
a – electrophoretic curve of mixture of dextran fractions (mol. wts. 58,000 and 19,200) after 9,500 sec.
b – of mixture of dextran fractions (mol. wts. 52,000 and 18,000) after 10,600 sec.
c – of mixture of dextran fractions (mol. wts. 36,000 and 18,000) after 17,000 sec.
d – of mixture of dextran fractions (mol. wts. 48,000 and 29,000) after 15,000 sec.

TABLE 2

Electrophoretic Separation of Mixtures of Dextran Fractions.

Molecular wt. of dextran fraction	Composition of mixture			Results of electrophoresis			Yield of fractions in %	u of fraction / u of fraction in mixture
	Concentration in g/ml	% of fraction in mixture	u of fraction in mixture in $cm^2 v^{-1} sec^{-1} 10^{-5}$	Concentration in g/ml	% of fraction in mixture	u of fraction in mixture in $cm^2 v^{-1} sec^{-1} 10^{-5}$		
58,000	0.005	33.3	1.45	0.0054	36.3	1.34	109.6	1.08
19,000	0.01	66.6	2.30	0.0096	63.7	1.95	95.8	1.18
52,000	0.01	50.0	1.58	0.0093	46.7	1.61	93.4	0.98
18,000	0.01	50.0	2.32	0.0107	53.3	2.42	106.6	0.96
36,000	0.005	33.3	1.91	0.0060	39.8	1.92	119.5	0.99
18,000	0.01	66.6	2.32	0.0090	60.2	2.42	90.4	0.96
48,000	0.1	50.0	1.65	–	–	1.70	–	–
29,000	0.1	50.0	2.05	–	–		–	–

DISCUSSION OF RESULTS

Both the results given in the present paper and the data of Northcote [6] demonstrate that polysaccharides, composed only of glucose, display in borate buffers characteristic electrophoretic mobility. This fact, however, is not in agreement with the accepted theory of the mechanism of reaction of borate with polyhydroxyl alcohols. Glucose residues do not contain hydroxyl groups in the cis position, with the exception of the outer glucose with free C-1. But the electrophoretic mobility of dextran is not the result of formation of only one strongly electronegative complex in the outer glucose residue in the chain. This is demonstrated by the determination of "valence", that is, of the amount of ionizing groups of the dextran-borate complex.

Two homogeneous dextran preparations – one of molecular weight 36,500 and the other of 29,000 were analyzed electrophoretically in 0.025, 0.05 and 0.2 M borate buffers, with determination in the same medium of the diffusion constant, resulting viscosity and partial molar volume. For determination of the molecular characteristics of these same dextrans, but not complexed with borate, corresponding analyses were conducted in 0.15 M NaCl, brought to pH 10 with sodium hydroxide. The results obtained are given in Table 3.

TABLE 3

Physico- and Electrochemical Constants of Borate-Dextran Complexes.

Mol. wt. of dextran	Molarity of borate	$D^{25°}_{H_2O} \times 10^7$	Mol. wt. of complex	l/d	$a \times 10^{-5}$, cm²V⁻¹sec⁻¹	Charge for spherical particle in coulombs × 10^{13}	Valence for ellipsoid
36 500	0,2	5.00	75 300	14	2.20	37.28	49.55
	0,05	5.42	61 200	14	1.58	16.83	23.34
	0.025	6.65	42 600	12	0.20	1.48	1.94
	0,0 (0,15 *M* NaCl, pH 10)	6.53	36 500	14	0.0	0.0	0.0
29 000	0,2	6.18	59 700	10	2,20	25.44	32.75
	0,05	6.43	46 100	12	1.75	12.51	15.64
	0,025	7.41	34 900	11	0.20	1.00	1.28
	0,0 (0,15 *M* NaCl, pH 10)	7.50	29 000	12	0.0	0.0	0.0

Assuming a spherical form for a molecule of dextran-borate complex, the charge of this molecule (in coulombs) can be expressed by the formula:

$$Q = \frac{6\pi\eta r\,(1 + \varkappa r + \varkappa r_i)}{f(\varkappa r)\,(1 + \varkappa r_i)}\,u. \qquad (2)$$

The charge in coulombs, multiplied by $300/4{,}8 \times 10^{-8}$, gives the amount of unit negative charges on the molecule. In the above formula u – rate of movement in $cm^2 v^{-1}$ sec $^{-1}$, κ – reciprocal of thickness of the double layer, taken as 0.411 A, r_i – average radius of ions in the medium, taken as 2.5 A, r – radius of the assumed spherical particle, calculated from the diffusion constant by the formula:

$$r = \frac{kT}{6\pi\eta D}, \qquad (3)$$

where k – Boltzmann's constant; other symbols are the same as in formula (1) [10]. But the dextran molecule is not spherical, and its degree of deviation from the spherical form can be determined by comparing the radius of a hydrated sphere, calculated from the diffusion constant (3), with the radius which would correspond to a spherical particle of molecular weight M and density $1/\bar{V}$(1). As the result of this type of calculation the asymmetry values (1/d) of the dextrans and dextran-borate complexes are obtained, which are given in Table 3. Knowing the asymmetry values and using the table of Abramson et al. ([10], p. 132), it is possible to convert the "valence" of a spherical molecule to the valence of the actual ellipsoid particle; these calculations are given in the last column of Table 3.

TABLE 4

Binding of Borate by Dextran in Possible Types of Complexes.

Type of Borate residue	Boron in moles per mole of glucose	
	Dextran M 36,500	Dextran M = 29,000
$B(OH)$	6.03	6.22
$B(OH)_2$	3.75	3.87
$B(OH)_3$	2.72	2.81

These data show that in the entire range of borate concentrations studied, the average amount of charges of the borate-dextran complexes is greater than 1, and that it increases gradually with increase of borate molarity. Hence it follows that there enters into the reaction with borate not only the outer pair of hydroxyl radicals of the dextran chain, but many more.

The amount of borate residues, which can be bound by dextran, is a function of its molecular weight, since each glucose residue in the chain with 1, 6-glucoside bonds has three free hydroxyl groups. Lateral chains, having these same bonds, block the hydroxyl group at C-4 of the corresponding glucose residues of the main chain, but, having a free OH-group at C-6 of its outer glucose residue, they do not change the average value – three OH-groups for every glucose residue of dextran. The presence of the two additional OH-groups of the outer glucose residues of the main chain do not essentially influence this value in dextrans of molecular weight about 30,000 (about 200 glucose residues in the particle).

From the above it follows that maximal saturation of dextran with borate can not exceed 3 borate residues per 2 glucose residues, assuming that the reaction leads to formation of a compound of the A or BD type (see introduction). Steric conditions make less probable complexes of the BD (and BD_2) type, in which tetracovalent borate reacts with an angle between valences of 109° [1]. Complexes of trivalent borate (120° angle between valences), that is, compounds of the A type, could nevertheless be formed, especially in a helical dextran molecule [7].

Here it should be recalled that complex formation between two molecules of dextran and one borate ion (the so-called borate bridge), as Zittle [2] assumes, is excluded in view of the possibility of electrophoretic separation of a mixture of different dextran fractions and in view of the existence of the identical electrical mobility of the products of separation and of the original fractions.

The amount of borate residues, bound to dextran, can be calculated by measuring the molecular weight of dextran and of the corresponding borate-dextran complex. The data relating to this are given in Table 3. The difference between these weights is due to addition of borate particles and elimination of a corresponding amount of water particles.

The reaction of borate with a glucose residue consists essentially of addition of a borate residue of over-all formula $B(OH)$ or $B(OH)_2$ to a glucose anhydride residue, a part of whose hydroxyl groups are deprived of hydrogens (see above – formulas A and BD). Thus, deduction from the molecular weight of the borate-dextran complex of the molecular weight of the dextran minus 3 (three hydrogen atoms) for each glucose residue in the particle gives the increase in weight, corresponding to the sum of added borate residues. Hence, by taking the molecular weight of the corresponding borate residue and the amount of glucose residues in the dextran particle, the amount of boron ions, bound by one glucose residue, can be calculated. Results of such calculation for borate-dextran complexes, formed in 0.2 M boric acid solution of pH 10, are given in Table 4.

From Table 4 is seen that formation of complexes of the A or BD type is impossible, since instead of the maximally permissible amount of 1.5 borate residues per 1 glucose residue is found for two different dextrans the value ~ 6 (for $B(OH)$) and ~ 4 (for $B(OH)_2$). The results obtained comply with the hypothesis that each borate residue reacts with only one hydroxyl group and maximal saturation is equivalent to 3 borate residues per 1 glucose residue. Saturation close to this value is given by the calculation based on the assumption that a trivalent borate residue ($B(OH)_2^-$) enters into the reaction, and a value identical to this is given by the calculation based on the assumption that to each OH-group of glucose is bound one residue of tetracovalent borate ($B(OH)_3^-$). However, the low degree of dissociation of boron residues bound to dextran (~0.4% at pH 10) makes this second possibility less probable.

SUMMARY

Homogeneous dextran fractions have in borate solution of pH 10 an electrophoretic mobility proportional to the molecular weight of the dextran within the limits 18,000-60,000.

Rate of electrophoretic movement of dextran depends on molarity of the borate. The greatest difference in rate of different dextran fractions is observed in 0.045-0.085 M buffers. Both in more and in less concentrated solutions of boric acid this difference decreases.

The possibility has been established of electrophoretic separation of mixtures of dextrans, differing in molecular weight.

The number of bound borate residues considerably exceeds 1.5 per glucose residue, which excludes the possibility of formation of cyclic borate-diol compounds.

The experimental data obtained best comply with the hypothesis that each hydroxyl group of glucose is bound upon saturation to one borate residue.

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SPECIES DIFFERENCES IN CHEMICAL STRUCTURE OF GLYCOGENS

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Concepts of glycogens, as of polyoses, with strongly branching chains of glucose radicals, bound by α-1,4-glucoside bonds, and at branching points by α-1,6-glucoside bonds, have been obtained, as is known, chiefly on the basis of work of Haworth [1] and K. Meyer [2, 3], who investigated in detail the glycogen of molluscs (the latter are a convenient source of glycogen since they contain 14% of the dry weight). On the basis of certain facts and especially of recent work of Cori's school [4] of the two schemes of glycogen structure – the laminar of Haworth and the branched of K. Meyer [3] – the latter is to be preferred.

The following questions, however, are quite pertinent: 1) can Meyer's scheme, proposed on the basis of study of glycogen of only one animal species, be accepted for glycogens of all other animals? 2) if species differences in glycogens exist, in what do they consist?

In spite of the many studies which indicate the differences and variability of glycogens (see for example [6, 7]), direct data on chemical differences in glycogens are not numerous. Until recently there have been found in the literature, as a rule, either data on molecular weights of various glycogens, or (see for example [8, 9]) some property such as "chain unit" (i.e., the number of glucose radicals belonging to one terminal nonaldehyde radical). Inasmuch as these studies have usually been made by various authors, comparison of results is difficult. It must likewise be emphasized, that the structure of the central part of the glycogen molecule, its "nucleus", formed after splitting off of peripheral chains of glucose residues by β-amylase, has been studied only in glycogens of molluscs [3] and rabbits [4], and here the problems were different and different methods were used. This makes impossible a comparison of the data obtained with respect to species differences. The type of bond at branching points was also studied in only two glycogens [1, 3], although the question is of great interest.

To answer the question raised, we made a detailed comparative study of liver glycogen of the rabbit and frog.

The choice of material was dictated by the following considerations: 1) Inasmuch as these animals represent both warmblooded and coldblooded, greater differences in the glycogens might be expected; 2) we had studied both glycogens previously with respect to the iodine reaction and its changes during gradual shortening of the external branches by β-amylase [10, 11]; 3) glycogen of the frog – an animal so often used for experiment – is at the same time one of the least studied.

Information in the literature has left no doubt that the structural unit of these glycogens is α-glucose. α-Amylolysis with formation of maltose indicated that the most often recurring bond in these polysaccharides is the α-1,4-glucoside bond. β-Amylolysis, leading to formation of β-dextrins, indicated the presence of a branched chain in molecules of both glycogens.

Thus, for chemical characterization of glycogens it was necessary to establish: molecular weights, cleavability by amylase with formation of β-dextrins, average chain unit of glycogens and of their β-dextrins and the type of glucoside bond at branching points. On the basis of the data obtained, average lengths of external and internal branches of the glycogen molecules could be calculated.

Liver glycogen of rabbit and frog was obtained according to Ostern [13] and purified by reprecipitations with alcohol. The preparations obtained left no ash, contained 8-10% moisture, 0.025-0.1% nitrogen and indeterminate traces of phosphorus.

Determination of Molecular Weights of Glycogens

For determination of molecular weights we used the method of Meyer [14], which consists essentially of oxidation of the polysaccharide with dinitrosalicylic acid and photometric estimation of the reaction products in comparison with products obtained upon oxidation of maltose. This method, no less accurate than physicochemical methods, has been successfully applied in recent years in polysaccharide chemistry.

All polysaccharide preparations were carefully dialyzed in the presences of toluol and, since they were used not only for determination of molecular weights but for other studies as well, precipitated with 3-4 volumes of aldehyde-free ethyl alcohol.

For each of parallel determinations dialysis was carried out separately, which served as one means of checking the method. After filtration through a hard filter (Wattman No. 50) and drying the precipitate over P_2O_5 a part of the preparation was hydrolyzed by acid and the polysaccharide content found from glucose determination in the hydrolyzate (moisture determination is a less accurate method).

In accordance with Meyer's method a standard curve was constructed for oxidation of maltose (in the amounts 0.2-2.0 mg) by 3,5-dinitrosalicylic acid. The latter (m.p. 174°) was synthesized according to Sumner [15].

Batches of glycogen (200-250 mg, dry weight) were dissolved in 2 ml water and to each solution were added 1 ml 1% 3,5-dinitrosalicylic acid solution and 1 ml 6 N NaOH, as in construction of the standard curve. The reaction mixture was kept at 65° in a water bath for 30 minutes. The control mixture was a solution of glycogen in 3 ml water and 1 ml 6 N NaOH, to which after 30-minute heating and subsequent cooling 1 ml dinitrosalicylic acid solution was added. Experimental and control solutions after cooling were diluted with water to 24 ml and subjected to photometry in a Pulfrich photometer with 200-mm cuvette and dark green light filter (λ = 500 mμ). Then from the standard curve the amounts of maltose were found to which the extinctions obtained in the experiments on oxidation of glycogens (and their β-dextrins) corresponded.

Molecular weights were calculated according to the formula $M = 2 \cdot 162 \frac{a}{b}$, where 2 is the degree of maltose polymerization, 162 the equivalent weight of a glucose radical, a the content of glycogen or β-dextrin and b the amount of maltose found for a given experiment from the standard curve (i.e., containing the same amount of aldehyde groups as the given weight of glycogen [1]).

Variation in duplicate determinations was ~9%, indicating good reproducibility of the method (it is known that in determination of molecular weights of high polymers very large variations are usually observed).

For an additional check of the method we used the following means. Molecular weights of dextrins can be calculated on the basis of the molecular weights found for the original glycogens and the degree of cleavability by β-amylase of these glycogens. Comparison of the molecular weight values for β-dextrins found experimentally with the calculated ones shows them to be close (Table 1).

In Table 1 for each preparation two values for the molecular weights are given, representing the extremes of variation in repeated determinations. Values for the molecular weights found for various glycogens are 475,000-1,134,000, which is close to the values found by Oakley and Young [16] for rabbit liver glycogen by the osmometric method. We found no differences in the values for molecular weights of glycogens of rabbit and frog.

Determination of Cleavability by β-Amylase and Formation of β-Dextrins

The determination of cleavability of branched polysaccharides by β-amylase provides important data on the ratio of glucose radicals in the external branches and internal part of the molecule.

The needed β-amylase was isolated from soybean flour; preparations were carefully freed of admixtures of α-amylase and maltase. Determination of cleavability was carried out under conditions optimal for β-amylase activity.

TABLE 1

Average Molecular Weights of Glycogens and Their β-Dextrins

Preparation	Average molecular weight	
	found	calculated
Glycogen of rabbit liver Oct. 26	511,000	–
	475,000	
Its β-dextrin	265,000	250,000
	232,000	
Glycogen of rabbit liver (partially hydrolyzed)	247,000	–
	225,000	
Its β-dextrin	115,000	115,000
	112,000	
Glycogen of rabbit liver Oct. 23	1,134,000	–
Glycogen of frog liver Nov. 20	485,000	–
	522,000	
Its β-dextrin	239,000	262,000
	304,000	
Glycogen of frog liver Feb. 17	662,000	–
Glycogen of frog liver Feb. 23	1,009,000	–

As the data indicate, as shown in part in Table 3, cleavability values for glycogens of rabbit and frog are close (47-51%), which makes impossible a definite conclusion concerning differences in ratio of glucose radicals in the external chains and internal part of molecules of frog and rabbit glycogens.

β-Dextrins were isolated by alcohol precipitation and carefully purified by repeated reprecipitation. We were convinced of the completeness of cleavage in each preparation on the basis of control experiments (action of fresh portions of enzyme on the purified β-dextrins isolated).

Determination of Average "Chain Unit"

Determination of average "chain unit" was carried out by end-group analysis, made by the method of periodate oxidation, which is not less sensitive than the method of methylation with the expenditure of much less polysaccharide and time [17].

The method is based on the formation of one molecule of formic acid as a result of oxidation from each non-aldehyde terminal glucose radical of the polysaccharide. In the case of branched polysaccharides, the formic acid formed from an aldehyde glucose radical is considered negligible, and the number of formic acid molecules formed is considered equivalent to the number of non-aldehyde terminal glucose radicals in the original polysaccharide.

We used the technique of Potter and Hassid [18] as a basis. To check the periodate action and to determine the time necessary for glycogen oxidation, maltose oxidation was first carried out (as a lower representative of the polymer homolog series of the polymaltoside type). 220 mg maltose (dry weight) was dissolved in 10 ml 3% NaCl, 10 ml 0.35M $NaIO_4$ added and the mixture put in the icebox at 4-6°. After definite time intervals 1 ml samples were removed, in which excess periodate was decomposed by addition of a few drops of ethylene glycol, and the formic acid was titrated with 0.01 N NaOH and indicator: methyl red + methylene blue. Control samples (without maltose) were titrated in parallel. In conformity to theory, oxidation was practically complete when formation of three moles of formic acid had occurred from each mole of maltose. This took place after 25 hours; during the subsequent 25 hours there was no increase in formic acid, indicating the absence under our conditions of the process of "superoxidation" [17].

Oxidation of glycogens and their β-dextrins was conducted under analogous conditions. As in experiments with maltose, oxidation of these polysaccharides was practically complete in 25 hours.

The amount of formic acid found on titration (minus control) was converted to the number of end-groups in the sample, and from this the average value of the "chain unit" was determined (Table 2).

TABLE 2.

Average "Chain Unit" of Glycogen Preparations

Glycogen preparation	Sample in mg (dry weight)	0.01 N NaOH in ml (minus control)	"Chain unit" (no. of glucose radicals)
From rabbit liver Oct. 26	108.2	0.481	13.9
" " " Oct. 26	104.0	0.455	14.0
" " " April 16	112.1	0.533	13.0
" " " April 16	107.5	0.507	13.0
" " " April 16	60.0	0.47	13.3
" " " Oct. 23	58.5	0.48	13.4
From frog liver Feb. 20	104.9	0.55	10.7
" " " Nov. 20	104.2	0.559	11.0
" " " Nov. 20	104.2	0.546	11.7
" " " Jan. 8	100.8	0.546	11.5

Variation in values, found in duplicate determinations, is 0.5-0.7 of a glucose radical. The values obtained for various glycogen preparations of each species of animal showed somewhat greater variation; however, the chain unit of rabbit glycogen was, as a rule, equivalent to 13-14 glucose radicals, and that of frog glycogen to 10-12 radical

In determination of the "chain unit" of β-dextrins, data for which are in part presented in Table 3, we also found that in frog dextrins "chain units" were shorter than in those of rabbit glycogen.

Type of Glucoside Bond at Branching Points of Rabbit and Frog Glycogens

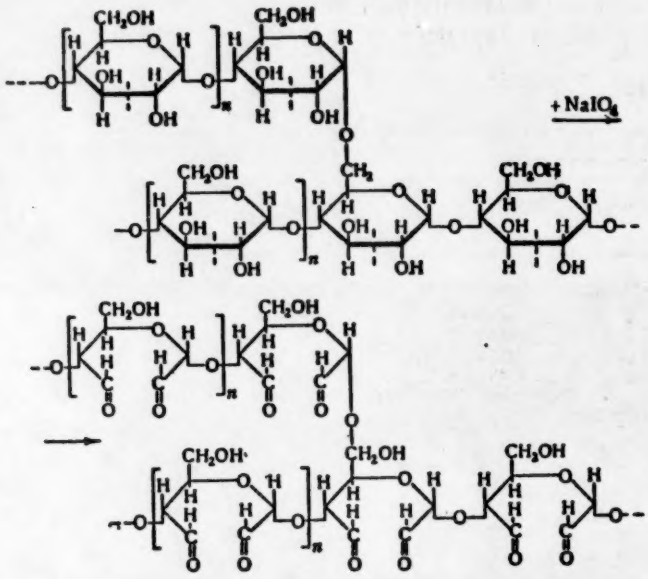
In order to answer the question, whether glucose radicals in rabbit and frog glycogens are bound at branching points in the same way as in mollusc glycogen, i.e. by a 1,6-glucoside bond, or whether they contain 1,2- or 1,3-bonds, the methylation method could be used.

In recent years, however, for this purpose the much less laborious method of periodate oxidation with subsequent chromatographic analysis of the hydrolyzate has been widely used. Thus, for example, the bonds at branching points of a number of amylopectins [19] were studied by this method. The principle of such analysis is based on the following: If in the polysaccharide there are only 1,4 and 1,6 bonds, then under periodate action on all glucose radicals, including those at branching points, bonds between carbon atoms 2 and 3 are broken (inasmuch as they are bound to free hydroxyls).

In this case after hydrolysis of the oxidized glycogen, glucose can not be found in the hydrolyzate.

But if in the polysaccharide there are 1,2 and 1,3 bonds at branching points, then under periodate action on the glucose radicals, occurring at branching points, there does not occur a breaking of bonds between carbon atoms (since in this case there are not present two free, adjacent hydroxyls), and the glucose radicals, occurring at branching points, will be retained under periodate action, and after hydrolysis of the oxidized glycogen glucose can be found.

Periodate oxidation of glycogen was carried out essentially by the method of Potter and Hassid [14]. The oxidized glycogens were dialyzed for 52 hours in the presence of antiseptic, then precipitated by alcohol and dried. Hydrolysis of preparations was conducted with 2.5% HCl for 3 hours. Study of hydrolyzates was carried out in two ways. In some cases HCl was neutralized with NaOH and then chromatographed with the use as an indicator of reliability not only of pure glucose but also glucose in NaCl solution, which excluded the effect of



Scheme of oxidation of polysaccharide with 1,6 bonds at branching points.

sodium chloride on the separation. In other cases the acid hydrolyzates were passed through a column of anionite, as a result of which the chlorine ion is replaced by OH^- (completeness of exchange was checked by the silver nitrate test). The hydrolyzates neutralized or treated with anionite, containing ~10 mg/ml oxidized carbohydrates (calculated on the initial polysaccharide), were concentrated 10-15 times by repeated application and drying of drops directly on the paper (or after preliminary evaporation under vacuum). In all cases Wattman No. 2 paper was used for chromatography with phenol-water as solvent. In some cases an ammoniacal silver nitrate solution was used as developer, in others, the more sensitive aniline phthalate.

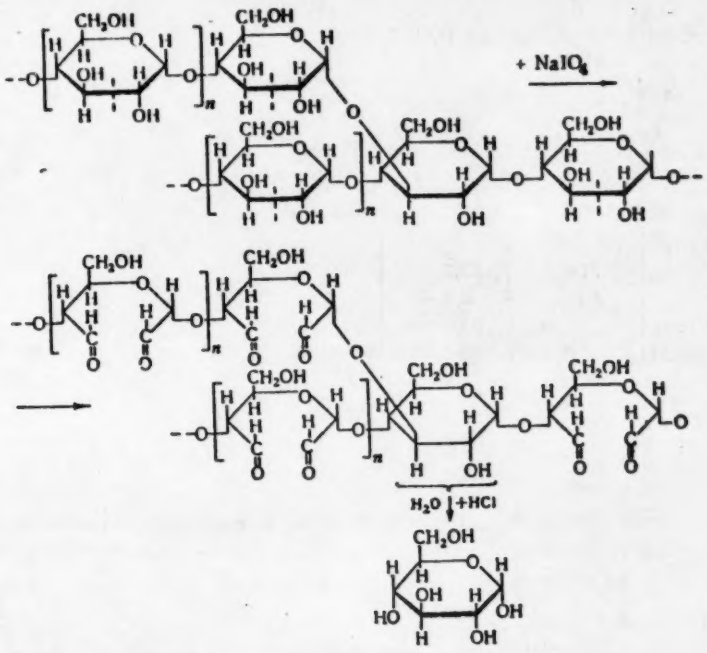
Preliminary control experiments showed that under the given conditions glucose in 0.1% concentration appears very clearly. Under the same conditions in hydrolyzates of oxidized glycogens of rabbit and frog, glucose could not be found in a single experiment. These results show that if 1,2 or 1,3 bonds are contained in the glycogen molecules, the amount is less than 0.6% of other bonds (1,4 and 1,6).

Thus, with respect to the type of bond at branching points, the glycogens of rabbit and frog do not differ from that of molluscs.

Structure of Glycogens of Rabbit and Frog

The data obtained in the preceding sections provide a conception of the structure of the glycogen molecules of rabbit and frog. Obviously these polysaccharides have a number of features in common: the order of magnitude of their molecular weights is the same; the ratio is approximately the same of glucose radicals contained in external branches, to radicals contained in the internal part of the molecule; in both glycogens there are 1,6-glucoside bonds at branching points; if there are other bonds (1,2 or 1,3) at branching points, their number does not exceed 0.6% of the total number of bonds. Thus, in general type of structure the two glycogens are rather close.

At the same time these glycogens also have essential differences: the average value of the "chain unit" of rabbit glycogen is greater than that of frog glycogen and, although the differences are not great, they exceed the limits of accuracy of the method and are constant.



Scheme of oxidation of polysaccharide with 1,3 bonds at branching points.

Knowing the value of the "chain unit" and cleavability by β-amylase, the average length of external branches of the molecule can by calculated:

$$N_E = \frac{N \cdot \beta}{100} + 1.5 ,$$

where N_E is the average length of external branches; N the value of chain unit; β the cleavability by β-amylase in percent and 1.5 the average length of "stumps", i.e. radicals of external branches remaining in β-dextrins after action of β-amylase (according to the literature, after β-amylase action on glycogen 1-2 glucose radicals remain bound to each peripheral branching point, i.e. on the average 1.5 [8]).

The average length of internal branches can be calculated approximately by subtraction from the average "chain unit" of the average length of an external chain plus one (i.e. of a glucose radical, representing a branching point): $N_I = N - (N_E + 1)$. The lengths of internal chains thus calculated are given in the last column of Table 3 (in parentheses).

The special study of β-dextrins described above provided an opportunity to check these values, by calculating average lengths of internal branches by another formula:

$$N_I = \frac{n - a - (3 \cdot 0.5a)}{a} = \frac{n - 2.5a}{a} .$$

In this formula n the polymerization coefficient of β-dextrin (calculated from molecular weight value); a the number of branching points in the molecule (practically equal to the number of end-groups); the coefficient 3 the average number of glucose radicals remaining bound to each peripheral branching point after

TABLE 3

Data on Structure of Various Glycogens and Their β-Dextrins

Preparation	Polymerization coefficient (n)	Av. "chain unit" (N)	Av. no. of branching pts. in molecule (a)	Cleavage by β-amylase (β)	Av. length of ext. branches (no. of glucose radicals) N_E	Av. length of int. branches (no. of glucose radicals) N_I
Glycogen of rabbit liver 10/26	3170	13.5	228	48.7	8.1	(4.4)
Its β-Dextrin	1550	8.2	200	0	(1.5)	4.9
Glycogen of rabbit liver (partially hydrolyzed)	1450	14.0	103	51.0	8.6	(4.4)
Its β-Dextrin	700	–	103	0	(1.5)	4.3
Glycogen of frog liver 11/20	3100	11.0	281	47.5	6.7	(3.3)
Its β-Dextrin	1650	–	281	0	(1.5)	3.3

β-amylase action; 0.5a the number of all peripheral branching points (practically equal, according to the rules of dichotomous branching, to half the total number of branching points). Values for N_I, calculated by this formula, are given in the last column of Table 3 without parentheses.

As seen from Table 3, the values calculated by the two methods for average lengths of internal branches coincide or almost coincide. The data obtained, in part presented in Table 3, indicate that not only external but also internal branches of frog glycogen are shorter than those of rabbit glycogen.

On the basis of the data obtained diagrams can be "constructed" of sections of molecules of rabbit and frog glycogens (Fig. 1). We have given diagrams of sections the general form of a segment of a circle.

As a consequence of the presence of shorter internal branches the molecule of frog glycogen is constructed more compactly.

It should be mentioned that much earlier, on the basis of a study of the iodine reaction of glycogens and their changes upon β-amylolysis, we deduced the shorter length of external branches of frog glycogen [11, 12]. Now this deduction has obtained direct confirmation on the basis of a chemical study of glycogen structure. The indicated differences in structure are also manifested in the properties, in the first place with respect to the iodine reaction (Fig. 2).

Longer external branches of the molecule produce a different position of the absorption maximum. As we showed previously [11], cleavage of rabbit glycogen by β-amylase to the extent of 16.8% leads to displacement of the maximum to the same region where the maximum for frog iodine-glycogen occurs. 16.8% of the average length of external branches of rabbit glycogen, found by us in the present paper, represents 1-2 glucose radicals, which coincides very well with the difference we established in lengths of external branches of the glycogens studied.

At first glance it may seem strange that such small differences in average lengths of external branches of glycogens can so strongly influence the iodine reaction. However, if the absolute dimensions are compared of a glucose radical (0.65 mμ) and an iodine molecule (0.63 mμ), it becomes obvious that small differences in lengths of external and internal branches (equal to 1-2 radicals) can substantially influence iodine adsorption.

The differences we have found in the structure of various glycogens are probably not altogether fixed in a quantitative sense. However, in numerous studies presented only in part in this paper, we have never encountered in rabbits such short external and internal branches as in frog glycogen, and vice versa.

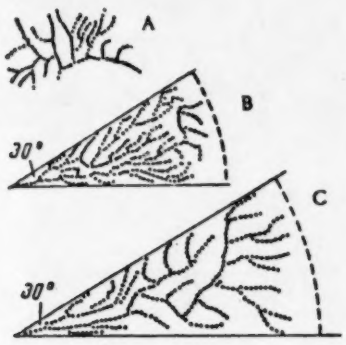


Fig. 1. Diagram of structure of glycogens: A – of molluscs according to Meyer [2]; B – of frog and C – of rabbit: Dots are glucose radicals. Diagrams B and C, representing a section of the molecule, contain about 300 glucose radicals; M is 162 × 300 = 48,000, which corresponds to the molecular weight of the whole molecule, 570,000. In diagram B the chain unit is 11 glucose radicals, average length of external branches 6-7 radicals, of internal about 3; in diagram C these values are 14, 8-9 and 4-5, respectively.

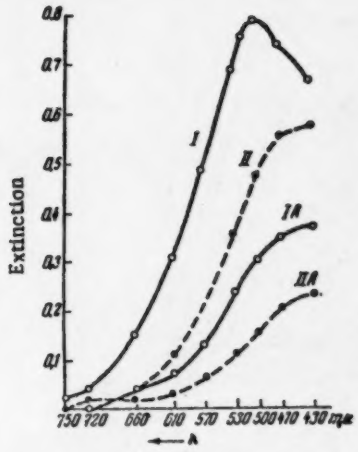


Fig. 2. Absorption curves of iodine compounds of glycogens and their β-dextrins: I – curve of iodine-glycogen of rabbit; II – same of frog; IA – curve of iodine-β-dextrin of rabbit glycogen; IIA – same of frog. 8.1 mg of polysaccharide dissolved in 25 ml phosphate buffer, pH 6.8. In cuvette: 5 microequivalents of I_2 and 2 microequivalents of polysaccharide glucose radicals.

SUMMARY

Liver glycogens of rabbit and frog and their β-dextrins were subjected to comparative study.

Determinations were made: of molecular weight by oxidation with dinitrosalicylic acid according to K. Meyer; of cleavability by β-amylase; of "chain unit" – a value which is the reciprocal of the degree of branching – on the basis of end-group analysis by the periodate method; of the type of bond at branching points by periodate oxidation with subsequent chromatographic analysis of hydrolyzates of the oxidized glycogens.

Average lengths of external and internal branches were found and diagrams "constructed" of the structure of sections of the glycogen molecules.

Glycogens of rabbit and frog are similar with respect to molecular weight, cleavability by β-amylase and type of bond at branching points; they contain 1,6-glucoside bonds, and if 1,2 or 1,3 bonds occur in molecules of the two glycogens, their number does not exceed 0.6% of other bonds.

Differences in the glycogens studied consist in the different degree of branching: frog glycogen has a shorter "chain unit", equal to 11 glucose radicals, average length of external and internal branches 6-7 and 3 glucose radicals, respectively, while rabbit glycogen is characterized by values equal to 13-14, 8-9 and 4-5, respectively. Thus, molecules of frog glycogen are more compact.

Good agreement was found between the structure of rabbit and frog glycogens and their iodine reaction, studied by us previously.

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ANTAGONISM BETWEEN SULFANILAMIDE AND FOLIC ACID IN GERMINATION AND CHLOROPHYLL SYNTHESIS OF WHEAT

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Utilizing germination of wheat as a means, proposed by Maevsky [1,2], of demonstrating the action of various agents on plant cells, we succeeded in showing that [sulfanilamide] at certain concentrations inhibits both growth of wheat sprouts and chlorophyll synthesis. Inhibition of growth of these sprouts by sulfanilamide preparations, according to data of Mednik and Braginsky [3], was removed by folic acid added to the nutrition medium, but not by p-aminobenzoic acid in concentrations corresponding to its portion of the folic acid molecule. The type of interaction between folic acid and sulfanilamide they did not explain. This was undertaken in the present paper.

EXPERIMENTAL

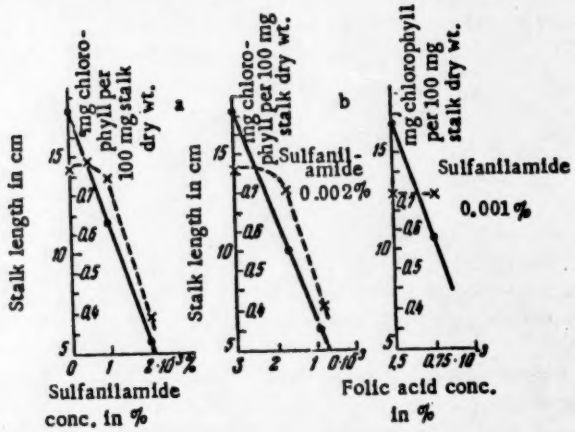
In all experiments wheat was grown in Petri dishes at room temperature; 20 grains of wheat were evenly distributed on gauze or cotton in ordinary tap water, to which was added sulfanilamide, folic acid or both in given proportions. For the first 24 hours the dishes were kept in darkness (to avoid folic acid decomposition before its absorption by seeds), then moved to a location uniformly lighted by diffuse daylight for 7-10 days. On completion of the experiment, always conducted under the same conditions with a control, the length of all sprouts in each dish was measured and the arithmetic mean value calculated of sprout length under the given conditions. Each experiment was conducted simultaneously in duplicate dishes. The amount of chlorophyll in sprouts was determined photometrically in photoelectric colorimeter FEK-M with red filter. Chlorophyll was extracted with ethyl alcohol from sprouts ground with quartz sand. The calibration curve was constructed from data for a solution of copper sulfate and potassium dichromate, according to the method of Hetry [4] (absorption intensity of a mixture of 28.5 ml 1% $CuSO_4 \cdot 5H_2O$, 50 ml 2% $K_2Cr_2O_7$ and 10 ml 7% ammonia, brought to 100 ml with water, corresponds to a chlorophyll solution of 8.5 mg % concentration). Dry weight of sprouts was determined by drying stalks in a drying cupboard at 105° to constant weight.

The effect of sulfanilamide was found to occur at concentrations in the solution of 0.002, 0.001 and 0.0005% and of folic acid at concentrations of 0.002, 0.0015 and 0.00075%.

In this table the results are shown of five series of experiments (arithmetic mean values ± standard deviation of the mean are given). As seen from the table, sulfanilamide at 0.002% concentration markedly inhibits wheat growth and strongly influences water content of sprouts (dry weight increased by 39%). There is an inverse relation between sprout growth and sulfanilamide concentration; the higher the sulfanilamide concentration, the less the growth. As seen from the figure (a), this is a straight-line relationship. Folic acid at 0.003% concentration in the absence of sulfanilamide does not affect growth, dry weight or chlorophyll synthesis of wheat sprouts. Sulfanilamide at 0.002% concentration markedly inhibits not only sprout growth, but also chlorophyll synthesis. Folic acid at 0.003% concentration completely removes the inhibition by 0.002% sulfanilamide on growth, chlorophyll synthesis and dry weight of sprouts.

Effect of Sulfanilamide and Folic Acid on Growth and Chlorophyll Synthesis of Germinating Wheat

Sulfanilamide in %	Folic acid in %	Stalk length in cm	Stalk dry wt. in mg.	Chlorophyll	
				mg/g stalk	mg/100 mg stalk dry wt.
0	0	17.3±0.28	150±5.9	1.14	0.76±0.02
0.002	0	5.5±0.65	208±5.4	0.83	0.40±0.04
0.001	0	11.5±0.72	151±4.0	1.12	0.74±0.02
0.0005	0	16.4±0.17	146±4.8	1.12	0.78±0.01
0	0.003	17.2±0.09	147±2.0	1.11	0.76±0.02
0.002	0.003	17.1±0.03	149±2.8	1.13	0.76±0.02
0.002	0.0015	9.9±0.88	157±3.4	1.10	0.71±0.02
0.002	0.00075	6.0±0.05	173±2.4	0.79	0.42±0.01
0.001	0.0015	16.6±0.26	156±5.5	1.10	0.70±0.02
0.001	0.00075	11.9±0.80	154±2.9	1.08	0.70±0.03



a. Effect of sulfanilamide on growth (continuous line) and on chlorophyll synthesis (broken line) of wheat sprouts.
Tangent of angle of slope of line – 2.9.
b. Antagonism of folic acid to sulfanilamide at 0.002 and 0.001% concentration of the latter.
Tangent of angle of slope of line at greater and lesser sulfanilamide concentrations, respectively 2.7 and 3.1.

Folic acid at lower concentrations also exerts appreciable antagonism on sulfanilamide with respect to removal of growth inhibition, chlorophyll synthesis and hydration of stalk tissues of sprouts.

Sprout growth in the presence of the two antagonists depends on the concentration of each. The relation of sprout growth to folic acid concentration in the presence of a given sulfanilamide concentration is also rectilinear (figure, b), and the slope of this line practically coincides with the slope of the line for relation of growth to sulfanilamide concentration (figure, a). This indicates that the action of folic acid and sulfanilamide is antagonistic and competitive. With respect to the interaction between sulfanilamide and folic acid in relation to chlorophyll synthesis, in this case we did not find a straight-line relationship. Only at 0.003% concentration does folic

acid counteract the action of 0.002% sulfanilamide, which inhibits chlorophyll synthesis. Folic acid at lower concentrations is inactive. With respect to the effect of sulfanilamide on the degree of hydration of stalk tissues of sprouts, folic acid in lower concentrations also exerts its antagonistic action, although in this case interaction between the two substances is noncompetitive.

Inasmuch as growth inhibition of wheat sprouts is directly proportional to sulfanilamide concentration, it is not difficult to calculate that in the concentration range we used the growth of 1 cm of sprout stalk is inhibited by 0.00017% sulfanilamide solution, and for removal of this inhibition folic acid is required in the solution in the amount of 0.00025%. If the molecular weight of these two antagonists is considered (288 for sulfanilamide and 441 for folic acid), then it is apparent that their competitive interaction occurs at a 1:1 ratio. Both solutions we used (0.002% sulfanilamide and 0.003% folic acid) are $6.8 \cdot 10^{-6}$ M.

The essential growth substances of every tissue are the nucleic acids and in particular ribonucleic acid, with whose participation protein synthesis, differentiation of tissues and every physiological orientation of living systems is accomplished [5]. This refers equally to animals and plants. Synthesis of nucleic acids, which contain adenine and guanine, is directly dependent on purine synthesis, from which both these purine bases are then formed. And for purine formation folic acid is required, which, taking up the product of intermediary metabolism of a number of substances, formaldehyde, gives it up as the second and eighth carbon atoms of purine. Sulfanilamide is evidently an antagonist not only of p-aminobenzoic acid, but also of folic acid, in whose molecule p-aminobenzoic acid occurs. In some unknown manner folic acid in the presence of the antagonist is deprived of the opportunity of taking up or transferring the one-carbon metabolite for purine ring formation. The purine synthesis, as well as the porphyrin synthesis, necessary for formation of the blood pigment heme and of the plant pigment chlorophyll, is accomplished according to the scheme of Shemin [6, 7] through formation from glycine and succinic acid of aminolevulinic acid and the hypothetical ketoglutaric aldehyde.

If sulfanilamide inhibits folic acid participation in the condensation process of the one-carbon radical, formed after decarboxylation of glycine and succinic acid, then the antagonism of these two substances – folic acid and sulfanilamide – is of the same type with respect to both growth and chlorophyll synthesis of wheat sprouts. Our experimental material does not indicate this. Evidently growth inhibition is accomplished by mechanisms participating in purine synthesis and inhibition of chlorophyll synthesis – by inhibition according to Shemin's scheme in the process of aminolevulinic acid formation.

SUMMARY

Sulfanilamide in 0.002% concentration strongly inhibits growth and chlorophyll synthesis of wheat sprouts. At this concentration of sulfanilamide the water content of sprouts decreases markedly.

Folic acid in 0.003% concentration completely removes the inhibition by sulfanilamide. At lower concentrations it removes the inhibition by sulfanilamide to a correspondingly less degree. The action of sulfanilamide and folic acid on growth of wheat sprouts is antagonistic and competitive at a 1:1 ratio. Inhibition by sulfanilamide of chlorophyll synthesis appears only at 0.002% concentration and is removed by folic acid at 0.003% concentration. At lower concentrations folic acid does not remove the inhibition of chlorophyll synthesis by sulfanilamide.

Inhibition of pyrrol synthesis by sulfanilamide apparently occurs through inhibition of formation of intermediate products, different from metabolic products leading to purine formation.

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AUTOLYTIC TRANSFORMATIONS OF GLYCOGEN DURING COOLING AND FREEZING OF MUSCLE TISSUE

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The autolytic and associated chemical and physicochemical changes in isolated muscle tissue largely determine the qualitative changes of meat and meat products during technological processing.

The first studies in this direction were made by Smorodintsev et al. [1]. Results of this work served as the basis for present conceptions of the nature of the maturing process of meat and as a beginning for further study of the chemical and physicochemical transformations occurring during other processes in meat processing.

Smorodintsev found that during storage of meat in carcass form under production conditions (4°), rapid glycogen decomposition and lactic acid accumulation occur in the muscle, reaching a maximum by 24 hours. Accumulation of inorganic phosphorus and displacement of the reaction of the medium to the acid side proceed simultaneously. These studies also showed that increase of residual and amino nitrogen does not occur in the maturing process. In addition Smorodintsev and Krylova [2] showed that peptones, products of beginning proteolytic decomposition of proteins, are absent in both fresh and matured meat.

Thus, the process of meat maturing is a stage of autolysis very far from that in which proteolysis and the associated tissue decomposition are most typical.

The numerous studies of Smorodintsev et al. indicate that meat maturing is caused by enzyme activity of the muscle itself [3]. In the initial stages of postmortem changes in muscle, glycogen and phosphorus compounds undergo profound transformation and protein substances change less markedly. As Drozdov and Zhuravskaya [4] have shown, in initial stages of muscle autolysis the parallel course of phosphorolytic (with further glycolysis) and amylolytic glycogen decomposition is typical.

Of especial interest was a study of autolytic changes in muscle at the negative temperatures at which a considerable amount of meat, designed for further processing, is actually kept; here the chief problem is the search for means of further improvement in freezing methods and the determination of optimal conditions for storage of frozen meat.

Although the work of the Drozdovs [5] and Smorodintsev [6] indicated the presence of enzymic transformations during freezing and storage of frozen meat, yet in most other studies dealing with freezing and thawing processes, the observed changes are related chiefly to ice crystal formation and histological changes, without sufficient consideration of the autolytic processes occurring in the muscle.

Among the intricate complex of changes occurring during refrigeration of meat, there is no doubt of the importance of following the autolytic transformations which largely determine the physicochemical changes that occur. Study of the biochemical changes, occurring during the entire cycle of meat refrigeration, is also important.

Autolytic processes in muscle associated with phosphorolytic and amylolytic glycogen decomposition and further glycolysis, i.e., with processes already occurring to a marked extent in the first hours after slaughter, are the actual beginning which predetermines the other postmortem changes that occur in muscle. Intensity of the course of glycolysis greatly affects the state of protein substances in muscle.

The Drozdovs [5] and Smorodintsev [6], studying accumulation of lactic acid and reducing sugars during meat refrigeration, did not make parallel studies of glycogen content.. Furthermore, in their experiments meat was frozen which was first stored for 24 hours at 4°. Under these conditions further changes in lactic acid content are quantitatively insignificant even at positive temperatures. Consequently, under such an experimental setup the glycogen transformations during cold treatment of muscle can not be judged very accurately. On the other hand, in the recent monograph of Smorodintsev [7] it is stated that glycogen, contained in unfrozen meat, is absent in the frozen. Data are completely lacking on enzymic glycogen decomposition during freezing of fresh (warm) meat.

This paper deals with the autolytic changes of carbohydrates of meat in the process of refrigeration at various temperatures, with the purpose of determining the nature of the enzyme reactions associated with muscle glycogen decomposition under these conditions.

Experiments were carried on the same group of muscles at various freezing temperatures. Thus, muscles frozen at −23° and −10° were stored at −18° and −10°, respectively. Muscles frozen at −35° and −10° were stored at the same temperatures. The experimental setup was based on the persistence of autolytic changes during the whole refrigeration cycle.

Within 1.5-2 hours after slaughter samples were taken to obtain data on fresh meat. Fresh muscles were refrigerated as well as those first stored 24 hours at 4°. In the latter case a muscle sample was taken before freezing for study of changes occurring as a result of autolysis during preliminary storage at positive temperature.

Immediately after freezing fresh muscles and those subjected to preliminary autolysis, samples were taken. To determine the autolytic changes occurring in frozen meat during storage at negative temperatures, samples were taken after one, two, four and six months of storage.

Samples for study were prepared in a room at −10°. A piece of frozen muscle was freed of the superficial layer and sawed in two. From the center of each piece a fine shaving was taken from which batches for determinations were obtained. For following the autolytic changes of muscle glycogen, known methods for determination of total glycogen, lactic acid, total reducing sugars and pH were used [8].

In order to avoid thawing of the material and to obtain unequivocal results, modification of these methods was necessary to meet the required demands. This was achieved by preparation of batches at low temperature (in a cold room), fine grinding and rapid application of reagents which produce immediate cessation of enzyme processes.

Results of the experiments showed that upon freezing fresh muscles (within 2 hours after slaughter) at −10° and at −23°, there are subsequently observed the simultaneous processes of glycogen phosphorolysis with further glycolysis leading to formation of lactic acid, and amylolysis with formation of reducing sugars (Fig. 1). Here we succeeded in showing very clearly the characteristics of the course of autolysis during freezing in comparison with analogous transformations at positive temperatures. With lowering of refrigeration temperature the rate of glycolytic processes is slowed and intensity of lactic acid accumulation is correspondingly reduced. There is simultaneous displacement of pH to the acid side.

On the other hand, accumulation of reducing sugars during freezing occurs considerably faster than at positive temperatures, increase in rate of accumulation of reducing sugars being greater at −23° than at −10°. Data from parallel studies indicate that during freezing accumulation of reducing sugars is much greater than during storage at 4° of the same muscle, obtained from the same animal, for the same length of time (Fig. 2).

At the same time it was found that during freezing of muscles, subjected to preliminary 24-hour autolysis at 4°, together with very insignificant lactic acid accumulation a distinct new formation of glycogen occurs (Fig. 2), somewhat more being formed at −23° than at −10°. During freezing, further accumulation of reducing sugars also occurs as in the previous experiments.

When fresh muscle frozen at −23° and −10° is stored at −18° and −10°, respectively, during the first month of storage the muscle glycogen decomposition and lactic acid accumulation continue, and from the end of the first month up to six months of storage there occurs a constant and continuous increase (new formation) of glycogen with insignificant lactic acid accumulation (Fig. 1). The relatively slow lactic acid accumulation during storage of frozen meat indicates the strong inhibition of glycolysis in frozen muscle.

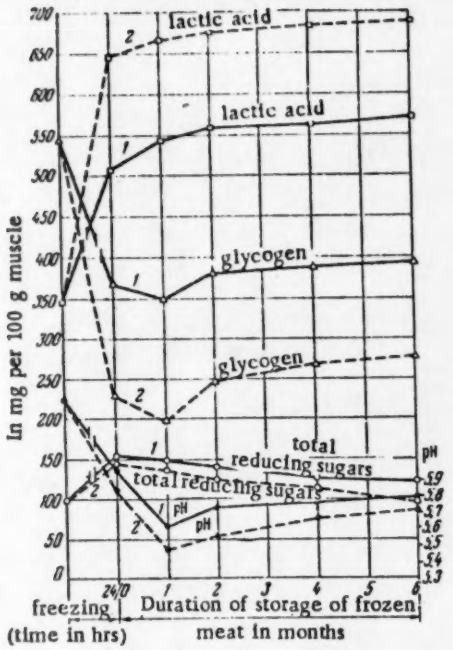

Fig. 1. Changes of pH, amounts of glycogen, lactic acid and reducing substances in muscle, taken 2 hours after slaughter, during freezing and storage in frozen state:
1 – Freezing at –23° and storage at –18°;
2 – freezing and storage at –10°.

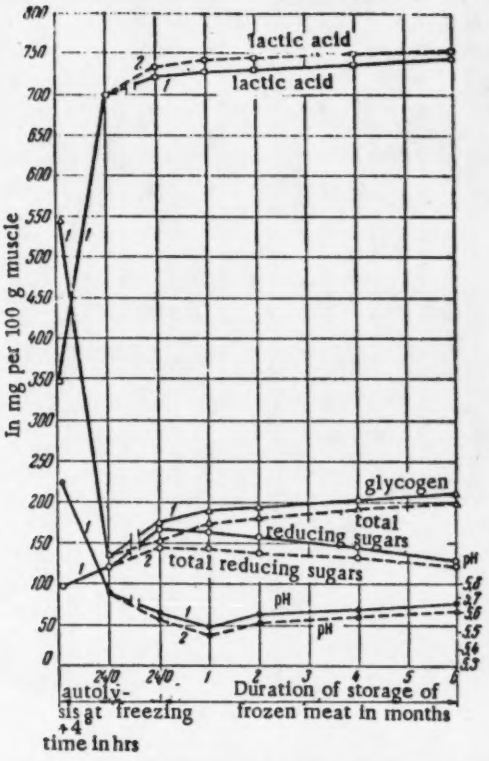

Fig. 2. Changes of pH, amounts of glycogen, lactic acid and reducing substances in muscle, first autolyzed 24 hours at 4°, during freezing and storage in frozen state: Symbols see Figure 1.

Parallel to and in conformity with the changes in lactic acid accumulation during the first month of storage of frozen muscle, pH displacement to the acid side is observed, these changes being the less, the lower the temperatures of freezing and storage of the frozen meat.

From the end of the first month up to six months of storage a certain increase in pH is noted. pH changes at different temperatures are practically the same. However, the pH value in muscles, frozen at –10° and stored at the same temperature to the end of the experiment, remains somewhat lower than pH of the same muscles frozen at –23° and stored at –18° (Fig. 1).

In muscles, subjected to autolysis prior to freezing, insignificant lactic acid accumulation occurs during the first month of storage and during subsequent storage (Fig. 2). New formation of glycogen, commencing in the muscles during freezing, continues during storage. In distinction to the changes observed during freezing, during storage of frozen muscles (with 24-hour and 2-hour preliminary autolysis), marked decrease in amount of reducing sugars occurs from the first month of storage.

The fact of new formation of glycogen during storage of frozen muscles is shown experimentally for the first time. A simultaneous decrease in amount of reducing sugars is observed, which is evidently due to the new formation of glycogen.

Next, experiments were conducted on freezing of meat at −35° and subsequent storage at the same temperature. In the literature it is stated that autolytic changes cease upon rapid freezing and are completely abolished at −30° [9]. The purpose of these experiments was to ascertain how correct this statement is and how autolytic changes occurring at −35° differ from those occurring at the negative temperatures used in the preceding experiments.

Experiments were also conducted at various negative temperatures. Samples from the same group of muscles (within 2 hours after slaughter) were subjected to freezing and storage at −35° and −10°. It proved that at −35° qualitatively the same autolytic changes are observed as with freezing at −10°: glycogen decomposition, accumulation of lactic acid and reducing sugars, pH displacement to the acid side (Fig. 3).

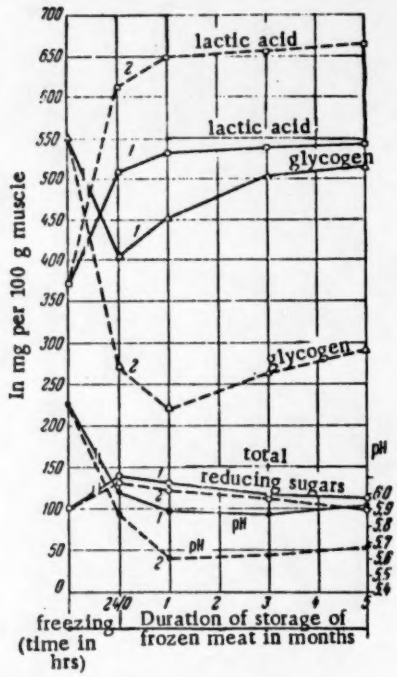

Fig. 3. Autolytic changes in muscle, taken 2 hours after slaughter, during freezing and storage in frozen state at −35° and −10°; 1 − freezing and storage at −35°; 2 − freezing and storage at −10°.

During freezing at −35° glycogen decomposition is strongly inhibited and new formation of glycogen in the frozen muscle at the same storage temperature occurs, commencing in the first month of storage. By the end of the fifth month of storage, the amount of glycogen in the muscle is close to its initial content in the unfrozen muscle. When the considerably smaller lactic acid accumulation during freezing and storage at −35° is also considered, it may be stated that in content of glycogen and lactic acid, muscle frozen and stored at −35° after 5 months of storage is closer to initial unfrozen muscle than that frozen and stored at −10°.

The principal results of the studies on autolytic changes, occurring in fresh muscles during cooling, freezing at −10°, −23° and −35° and storage at negative temperatures, are shown graphically in Figure 4. For comparison of the changes, experiments were chosen in which the initial muscles had approximately the same glycogen content. The data on the graph are differences, showing change in content of glycogen, reducing sugars and lactic acid for a given time in comparison with the initial data (initial data were subtracted from the experimental).

Results of our studies indicate the presence of continuous autolytic reactions in muscles, both at positive temperatures and during freezing and storage at various negative temperatures, and establish the qualitative and quantitative features of the glycolytic and amylolytic glycogen transformation under these conditions.

In contrast to the continuous glycogen decomposition during autolysis at positive temperatures, glycogen resynthesis is found at certain stages of the action on muscle of negative temperatures. These results show the relation of changes in reducing sugar content to those in glycogen content, the qualitative difference of the autolytic transformations of glycogen during storage of meat at negative temperatures, and the interrelation between increase (new formation) of glycogen, increase of lactic acid and decrease of amount of reducing sugars.

The effect of various temperatures of freezing and storage of frozen meat on its condition after six-month storage must be emphasized. As seen from Figure 4, contents of glycogen and lactic acid after 5-6 month storage at various freezing and storage temperatures differ sharply, the lower the temperature of refrigeration, the closer the meat is to unfrozen meat with respect to the features indicated.

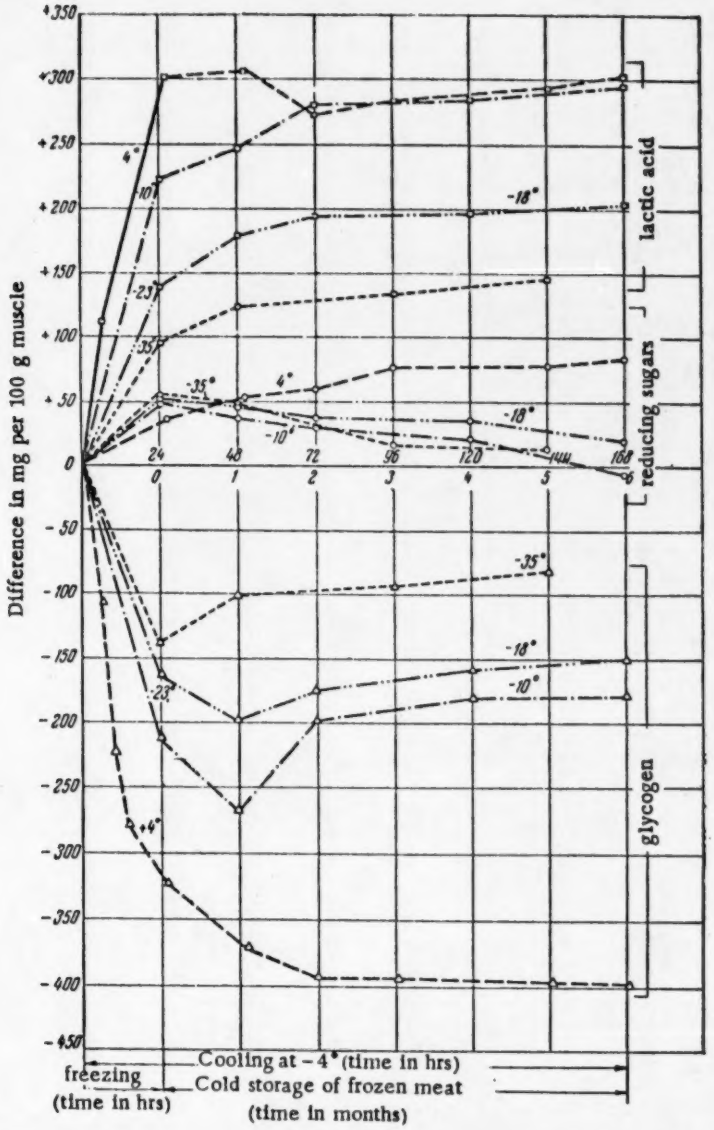

Fig. 4. Autolytic changes in glycogen, lactic acid and reducing sugars in muscles during cooling, freezing and storage at various temperatures.

The autolytic changes, occurring during storage of muscles frozen at various temperatures, do not correspond to those which occurred during freezing at various temperatures; lactic acid accumulation at negative storage temperatures is insignificant in comparison with its accumulation during the process of freezing.

SUMMARY

During freezing of fresh (warm) muscles at –10°, –23° and –35°, autolytic decomposition of muscle glycogen occurs at a slower rate, the lower the freezing temperature.

During freezing of fresh muscles and those first stored at 4° for 24 hours, lactic acid accumulates at a slower rate, the lower the freezing temperature, and reducing sugars – at a greater rate, the lower the freezing temperature.

During freezing and storage of frozen muscles subjected to preliminary 24-hour autolysis at 4°, new formation of glycogen is found, the amount being higher, the lower the temperature of freezing and storage.

During storage of frozen muscles, together with new formation of glycogen insignificant lactic acid accumulation is observed.

During storage of fresh muscles frozen at –23° and –10° glycogen decomposition and lactic acid accumulation are observed during the first month, and from the end of the first month up to six months of storage a constant and continuous new formation of glycogen. During storage of muscles frozen at –35°, new formation of glycogen occurs beginning in the first month of storage.

Parallel to new formation of glycogen during storage of frozen muscles, continuous decrease in amount of reducing sugars is found.

The autolytic changes in glycogen during refrigeration of muscles depend on the freezing temperature and do not equalize upon prolonged storage in the frozen form; here the type and rate of enzymic conversions of muscle glycogen depend on and are determined by the degree of autolytic changes in the muscle prior to freezing.

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HYDROXY ACIDS IN THE PRIMARY PRODUCTS OF PHOTOSYNTHESIS

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The study of the products of photosynthesis by means of carbon isotopes has shown that the labeled carbon of carbon dioxide is very rapidly converted in plants to a great variety of substances. Nevertheless the initial stages of this path, leading to formation of the primary products, are general ones, and only upon their further conversion are carboxylic acids, carbohydrates, proteins, lipids and many other substances obtained [1-3].

In the first studies with radioactive carbon it was found that the initial reaction of assimilation of carbon dioxide by plants is its entry into carboxyl groups of an unknown acceptor, this reaction being reversible [3]:

$$C^*O_2 + RH \rightleftarrows RC^*OOH.$$

Next, conversion of carboxyl groups to alcohol groups occurs in the light, accompanied by liberation of free oxygen:

$$RC^*OOH + H_2O \xrightarrow{h\nu} RC^*H_2OH + O_2.$$

These data were confirmed in subsequent studies and at present the various hypotheses concerning conversions of the primary products of photosynthesis are invariably based on substances containing both carboxyl and alcohol groups, that is, on hydroxy acids. However, concerning the nature of the primary acceptor of carbon dioxide there exist various views. According to some authors, this acceptor is a substance with two or five [4] and according to others with forty carbon atoms in the molecule [5].

Such hypotheses are usually based on the character of the products in which labeled carbon is found earliest in experiments with photosynthesis. Here much depends on the technique of isolating the primary products from the plant. Considering the rapidity with which the path of carbon is covered in photosynthesis, the desire of workers is natural to carry out this isolation as rapidly as possible, with use of very harsh methods. But then decomposition of products inevitably occurs and to judge by these concerning the primary acceptor and the further path of carbon has become impossible. This is why up to now it has been impossible either to isolate or even to find in the plant the fundamental oxidation-reduction system, which reduces carboxyl groups to alcohol groups.

Under milder treatment, without prolonged boiling with solvents, primary products of molecular weight greater than 1000 are usually obtained [5]. Our study of the elementary composition and of a number of properties of these products has shown that they are acids of molecular weight ~ 1500 (R'); from them trisaccharides (R''') are formed further in the process. According to the data on elementary composition and molecular weight, in the molecule of the primary product of photosynthesis there are found about forty carbon atoms, and in the trisaccharide molecule about eighteen. It remained to find into what functional groups the labeled carbon enters.

First determinations were made of the percent content of carboxyl groups, since from data obtained previously it was known that labeled carbon first enters these groups, and then passes into others. The percentage was determined by decarboxylation at 250° of dry barium salts of the isolated products, with subsequent absorption of the liberated carbon dioxide by barium hydroxide solution and titration with 0.1 N H_2SO_4 [3, 6].

Thus, it was found that the acid R' consists by weight of 30% carboxyl groups and that in these groups are found 25% of its carbon atoms. In the product R''' only about 16% carboxyl groups, containing 11.1% of the carbon atoms of the acid, was found.

Of further interest was investigation of the presence of alcohol groups in the primary products. They were determined by the method of benzoylation, with subsequent boiling of the products obtained with an alcoholic solution of sodium hydroxide and titration with 0.1 N H_2SO_4 [3, 6, 7]:

$$ROH + Cl - CO - C_6H_5 \rightarrow R - O - CO - C_6H_5 + HCl.$$

It was found that in R' alcohol groups represent 60% of its weight and they contain all the remaining 75% of the carbon atoms. Thus, the primary product of carbon dioxide assimilation in photosynthesis is a hydroxy acid containing in the molecule ten carboxyl and thirty alcohol groups. The determinations of the number of functional groups find confirmation in our data obtained previously [5] on elementary composition. In addition the molecule R' contains an atom of iron and an atom of phosphorus, the latter probably in the form of a phosphoric acid residue. The composition of product R' is given in Table 1.

TABLE 1

Composition of the Primary Product R'

Groups	% by wt.	no. in molecule	% C atoms in group
–COOH	30.0	10	25.0
–CHOH	60.0	30	75.0
Fe	4.0	1	–
$-PO_4$	6.4	1	–

TABLE 2

Composition of Product R'''

Groups	% by wt.	no. in molecule	% C atoms in group
–COOH	16.4	2	11.1
–CHOH	63.0	12	66.7
–CO–	5.0	1	5.5
Undetermined	15.6	3	16.7

In product R''' alcohol groups represent 63% of the total weight. The increase in its state of reduction in comparison with R' is chiefly due to decrease in percent of carboxyl groups.

After 3-hour hydrolysis with 2% HCl the capacity to reduce Fehling's solution appears in R'''; this is retained after treatment with iodine in an alkaline medium according to Kolthoff, which is supposed to destroy aldoses. Consequently, the reducing group here belongs to a ketose, which is confirmed by color reactions. The composition of product R''' is given in Table 2.

In product R''' iron and phosphorus were also found previously [5]. In order to determine its structure the solution obtained after hydrolysis with hydrochloric acid was precipitated with barium chloride and ketose determinations were made separately on substances in the solution and the precipitate. It was found that all the reducing substances remain in solution, and the barium precipitate is completely devoid of the capacity to reduce Fehling's solution. The substances present in it contained carboxyl groups, which appear prior to hydrolysis to R'''. These acids in both percent content of carboxyl groups and elementary composition were close to uronic acids. The composition of the hydrolysis products of R''' is given in Table 3.

Calculation of the amount of uronic acids was made from decarboxylation data. Product R''' is thus a trisaccharide, one-third consisting of ketose and two-thirds of acids of the uronic type, but not capable of reducing Fehling's solution and poorly decarboxylated according to Tollens. Hence it can be assumed that after removal of

TABLE 3

Hydrolysis Products of R'''

Composition in %	Before hydrolysis	After hydrolysis	
		Solution	Ba-precipitate
-COOH	16.4	0	15.4
"Uronic acids"	70.2	0	67.8
Ketoses	0	32.0	0

ketose from them by hydrolysis they remain in the form of diuronides, combined at the point of the reducing groups, and perhaps of the carboxyl groups. In recent studies dealing with metabolism of uronic acids in plants, similar diuronides and also other examples of the combination of uronic acids with a wide variety of substances were described [8].

According to data from experiments with labeled carbon, the product R''' is a precursor of free sugar formation in plants.

It is possible that during photosynthesis the formation of other substances, for example, of the protein or lipid type, begins at a still earlier stage – with conversion of the primary product – R'. Hence, investigation of its structure is especially important.

We succeeded in showing that upon decomposition of this hydroxy acid glycerol derivatives are formed. Thus, after 20-minute boiling with 0.3% solution of bromine water the reaction for dihydroxyacetone was very good with resorcinol and less good with β-naphthol. After careful oxidation of R' good reactions for glycerose were given [9]. Next, of especial interest was the performance with this altered product of Tollens' naphthoresorcinol reaction, characteristic of a whole series of hydroxy acids, including glyceric acids. It should be noted that none of these reactions is given by R' unaltered by previous treatments. The primary product does not give reactions for ketones, carbohydrates or hydroxy acids of the glyceric type. These substances are obtained only upon decomposition of R', which indicates the great possibilities for conversions of this product in different directions.

The appearance of glycerol derivatives upon decomposition of the primary product explains the reason for the finding by certain authors of labeled carbon earliest in glyceric acids [2, 4]. Under harsher conditions of isolation from the plant decomposition of R' occurred, and portions of its molecule were described as the primary products of photosynthesis.

The hydroxy acid R' contains double bonds, but an exact determination of the iodine number has not yet been successful, owing to poor solubility of R' in various solvents. Nevertheless, the values obtained for the iodine number were above 100. Complete solution in concentrated sulfuric acid is accompanied by disintegration with liberation of carbon dioxide. It is interesting to note that only under such conditions can the iron contained in this product be separated from the organic part of the molecule. Since the "glycerol part" of the molecule can be separated relatively easily, it must be assumed that the iron in R' is more firmly bound to the carboxyl groups. Also in favor of such a view is the ready loss of iron in cases of incautious decarboxylation and the data from experiments on the inhibition of initial carbon dioxide assimilation in photosynthesis by poisons, specific for suppression of reactions that occur with participation of metals. There are hypotheses in the literature [10] concerning a possible bond of iron with carboxyl groups in certain biochemical oxidation-reduction catalysts.

There occur in the primary product of photosynthesis ten carboxyl groups for one atom of iron. With respect to a number of properties its structure is similar to the recently synthesized iron-organic compounds of the dicyclopentadiene type [11, 12]. Some of them also contain hydroxyl groups; they are capable of many reactions of condensation and oxidation-reduction, leading to formation of diverse substances. The isolation from cells and determination of the composition of the hydroxy acids, appearing as the primary products of photosynthesis, has raised in turn the question of full investigation of their structure and conversions in photosynthesis, with the purpose of directing the reactions involved toward formation of the most desired products.

SUMMARY

In the primary products of photosynthesis R' and R''' isolated from plants, whose elementary composition and certain other properties we studied previously, determinations of carboxyl and alcohol groups were made.

The first of the products (R'), in which labeled carbon is found in photosynthesis, is a hydroxy acid containing in addition to carboxyl and alcohol groups, atoms of iron and phosphorus. These determinations of functional groups agree well with the elementary composition of this substance.

The product R'", in which labeled carbon is observed later in its path of conversion to sugars, also contains carboxyl and alcohol groups, but the percent of the former is considerably less compared to R'.

After hydrolysis it was found that one-third of it consists of ketoses and two-thirds of acids closely related in composition to uronic acids, but which do not reduce Fehling's solution and are decarboxylated with difficulty.

Upon careful degradation of the product R', products were obtained which give reactions for various glycerol derivatives: dihydroxyacetone, glycerose and glyceric acids. This indicates the great possibilities for conversion of the primary product in different directions. Furthermore, thus is explained why, under harsh condition of isolation from the plant, labeled carbon is found earliest in glyceric acids; they are obtained as a result of decomposition of the hydroxy acid R'.

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DISCUSSION

TWO FORMS OF SELF-RENEWAL OF PROTEIN

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The capacity for metabolism and self-renewal is the most significant property of living matter, and lies at the basis of its other properties, primarily the capacity for growth, variability and continuous development. Concepts of self-renewal of cellular proteins, of the "fluidity" of their chemical composition, of the presence of irreversible and of reversible breakdown of proteins were expressed long ago, but the most convincing evidence for these views became possible only in the last 15-16 years in connection with the extensive use in biochemistry of the method of labeled atoms.

During a relatively short period, since the first work of Schoenheimer et al. in this field, there have appeared hundreds of studies relating to various aspects of the problem of protein self-renewal. In proportion to the extent of study of these processes there has developed and continues to the present extensive discussion of the forms of protein self-renewal. The essence of this discussion is that some authors consider as the only form of protein self-renewal the alternation of processes of synthesis and breakdown of whole molecules in such a way that molecules of one type or another are synthesized, function for a certain length of time and then breakdown, being replaced by newly formed molecules, while other authors assume that together with this form of self-renewal, processes of partial renewal of molecules without complete breakdown are possible by means of replacement in macromolecules of individual amino acid and peptide groups.

In a study dealing with self-renewal and aging of proteins [1], we briefly considered the material of this discussion. However, in the interval new data in this field have accumulated, permitting a clearer and more definite conception of the biochemical character of protein renewal in the organism.

Metabolism and self-renewal are inseparable. But if by metabolsim is meant the total sum of chemical changes in the organism, then the concept "self-renewal" signifies those metabolic processes, which directly produce the fluidity, the "dynamic state" of the chemical composition of the principal structural components of living matter, foremostly of proteins and nucleic acids. "Self-renewal" is not something rigidly defined – the meaning inherent in this conception may change in relation to the object. Self-renewal of the cell consists of a great sum of processes in comparison with renewal of proteins, since it involves formation of carbohydrates, lipids and other substances. Self-renewal of the organism as a whole is still broader and more varied than self-renewal of a single cell. We are not concerned here with all the diversity of forms of natural renewal of the organism and will deal only with the question of the forms of protein self-renewal.

In living tissue new formation of proteins constantly occurs by means of synthesis with breakdown after a certain interval of time. The occurrence of these processes has been demonstrated, and it is not necessary to again recall the proof. This phenomenon of continuous replacement of molecular "generations" represents the basic form of biological self-renewal of proteins.

However, together with these processes a number of authors ([2-7, 22] and others) propose the occurrence in proteins of yet another form of self-renewal – the process of entrance into the structure of protein molecules and exit from them of individual amino acids and peptides, which is not accompanied by breakdown of the entire molecule as a whole or loss of specificity. This self-renewal represents an exchange without breakdown, indicating the lability of the internal structure of proteins in protoplasm. In the study referred to above we also subscribed to these views.

It must be noted, however, that while most proponents of these views consider that protein self-renewal without complete breakdown of molecules is possible only in a living system as a result of continuous and simultaneous action on proteins of various enzyme and energy systems, there are certain authors who attempt to represent this process as an inherent property of individual, pure proteins, which they can manifest also in an isolated state, outside the organism, separated from enzyme systems and energy exchange, and that thus individual chemically pure proteins are still alive [8-10].

This idea has been propagated particularly insistently in recent years by the journal "Problems of Philosophy". In a series of articles published in this journal on the question of protein self-renewal and origin of life [8, 11-16], a dogmatic and arbitrary interpretation is given of a number of notable sayings of F. Engels concerning protein. To every biochemist acquainted with the level of chemistry and biology of proteins in the second half of the last century, it is clear that Engel's concept of protein is applicable not to proteins characterized by means of methods developed several decades later, but to protoplasm as a whole, to intricate protein complexes, to the substratum which in reality under natural conditions is living, capable of growth, exchange, respiration, multiplication and irritability. Speaking of the properties of living protein, F. Engels had in mind the protein foundation of the cell protoplasm, and not those individual protein preparations of various enzymes, albumins, globulins, etc., isolated from intricate protein complexes by modern methods.

The journal "Problems of Philosophy" in elucidating the material of the discussion took, in our opinion, an incorrect position, by publishing in the course of the discussion a series of superficial articles misinterpreting the present state of the problem of exchange and self-renewal of protein and disorienting readers with respect to the facts obtained by the method of labeled atoms.

Concerned with such material are, for instance, the articles of Stukov and Yakushev [11] and Sysoev [15]. Explaining in a simplified way the modern factual material, Stukov and Yakushev write that "experiments with labeled atoms show that in living protein the atoms possess enormous mobility, as if they were chemically free within it and not confined by a chemical bond" (p. 144). The assertion is questionable, that "the molecule of living protein is conceived of not as a molecule in the physicochemical sense, but only as a quantitative measure of a chemical substance within the limits of which was produced and is now produced a conversion of dead organic matter to live organic matter. To the physicochemical concept of molecularity there corresponds here a concept of a discrete quantity of matter representing a measure within the framework of which a simple chemism is released, contradicting itself by the maintenance of this very chemical process" (p. 140). The authors arrive at the conclusion that "the modern chemical scheme of protein structure is not adequate, does not correspond to the level of modern biological knowledge, for it does not elucidate the basic living properties of the specific protein structure, the specific structure of the discrete protein molecule, does not show how protein spontaneously respires, is nourished, grows, divides and develops into protoplasm, a cell and an organism" (p. 149).

A speculative approach similar to this lies at the basis of the assertions of a number of other authors concerning the capacity of individual proteins for typical self-renewal, growth and development. As evidence of the correctness of these views are usually cited experiments of Konikova and Kritsman et al. [8-10] on "self-exchange" of isolated proteins. It is advisable in this connection to analyze thoroughly the actual value of this evidence.

Most of the experiments, described by Konikova, Kritsman and Osipenko [9], consisted of 24-hour incubation of radioactive methionine with plasma and pure proteins (globulins, albumin, pepsin, trypsin and others). Here they observed a certain incorporation of radioactivity into the precipitated proteins. But inasmuch as incubation in most cases was conducted under nonsterile conditions and without antiseptics, however, the authors defend the reliability of their results, the suggestion of microbiological contamination of the incubated mixture in these experiments, which was put forward and substantiated by Orekhovich et al. [17], remains valid.

In spite of criticism, Konikova, Kritsman and Samarina in a subsequent study [10] did not subject the original data to a careful check, but presented the results of experiments with other proteins. Most experiments consisted in incubation of a number of protein preparations with radioactive glycine at 100°. Under these conditions there was observed a quite appreciable incorporation of radioactivity into the protein preparations in the amount of approximately 1-2% of the total activity of the labeled amino acid. The result obtained, however, does not prove the biological self-renewal of isolated protein, since a certain addition of glycine-C^{14} to proteins occurred by reason of temperature modifications of the molecules (heat denaturation, etc.), having nothing in common with biological self-renewal. The possibility of physicochemical denaturation binding of amino acids was shown in recent experiments of Pasynsky et al. [18].

One experiment of the authors [10] was carried out at body temperature, incubation lasting for 2 hours, and consequently an appreciable development of microflora in this case could not occur. This is the only experiment in this work, not subject to doubt from a methodological point of view. Does it prove the "ability of isolated proteins to incorporate amino acids without participation of biological structures and added enzyme systems"? Unfortunately, it does not. Nine substances were studied in these experiments. Five of them can not possibly be considered to come under the category "individual" proteins, but represent protein complexes undoubtedly containing enzymes although in but slight admixture (rabbit liver homogenate, blood plasma and serum, hemolymph of oak silkworm, globulin complex of rabbit liver). The four others are nucleoproteins, and the method of isolation and purification not being given it is therefore hard to judge to what degree the preparations used for experiment were freed of admixtures.

When the numerical data of this experiment are considered, it is also impossible to find convincing results. For experiment were taken 25-100 mg of protein preparation and labeled amino acid with an estimated 100,000 counts/sample. The specific activity of the preparation and the ratio of labeled amino acid to the corresponding amino acid in the structure of the protein in mg or micromoles is not given, although this is of great importance in calculation of the degree of self-renewal in percent. Inasmuch as the reader himself must guess how much protein was used in each separate experiment, we will assume that for protein complexes the weight was 100 mg, for "individual" proteins 25 mg. Activity at the end of the experiment was calculated from weights of 5 mg and thus corresponded to 20,000 counts of the labeled amino acid added. In this case the difference between control and experimental protein amounted to that between duplicates: for pentosenucleoprotein of rabbit liver 1.3, 4.0 and 6.6 counts per preparation, that is, some hundredths of a percent of the activity of the original amino acid; for desoxypentosenucleohistone of rabbit liver 12, 15 and 13 counts, that is, also some hundredths of a percent (about 0.07%). Appreciable incorporation of activity (79, 86 and 74 counts per 5 mg of protein) was observed only in nucleoproteins of rat liver, the experiment with DNA of rat liver being carried out without duplicates and with appreciable activity in the control (10 counts/min.), comparable to the magnitude of "incorporation" of amino acids in the nucleoproteins of rabbit liver. Meanwhile, it is obvious that the slight activity could have appeared in the proteins through incidental reactions (combination of glycine with protein end-groups, action of slight admixtures of enzymes, reactions between chemically active groups of proteins or nucleic acids and the amino acid, contamination of the radioactive preparation, etc.). Such a possibility follows from the fact that in most of the controls in this and other experiments there is likewise a certain activity (0.4-10 counts/min per sample).

It must be noted that in the other protein substances (plasma, serum, etc.) used in this experiment, there was also observed only traces of radioactivity (0.5-18 counts/min per sample). To what quantity of labeled amino acid molecules these radioactivity values correspond, the authors nowhere indicate.

In their last article, published in the Journal "Problems of Philosophy" [8], Konikova and Kritsman presented a series of new experimental data on "self-exchange" of proteins and at the same time attempted to answer the criticism they had received. The new data presented by the authors show that decrease in concentration of the protein solution increases incorporation of labeled amino acid. Such a phenomenon is natural, since it is obvious that the less the protein concentration, the weaker the degree of aggregation of the protein complexes and the higher the quantitative ratio between "label" and protein. Details of technique are not described and therefore, it is impossible to make any sort of analysis of the numerical data in this article. The authors report the incorporation into proteins of the tripeptide glutathione. Interaction of similar type is represented by the usual chemical and physicochemical interactions of reactive groups, and an analogy between them and the actual processes of biological self-renewal of protein is extremely remote.

This was shown particularly clearly by Shnol [19], who found that in the interaction of free amino acids with protein preparations the binding of amino acids by free functional groups of protein occurs, but not an exchange in peptide chains, that is, not the "penetration" of amino acids into the structure of the primary chains, characteristic of living biological systems. Therefore, the conclusion of Konikova and Kritsman is unconvincing, that their data "show that self-exchange of simple proteins does not represent a replacement of like structural units of protein, but is an exchange which leads to recreation of proteins similar to themselves and proteins different from themselves, that is, an exchange that expresses the first biological form of movement of matter" (p. 174). The assumption that nonspecific combination of amino acids with free reactive groups of the protein molecules is basic to the solution of the complex problems of synthesis, reproduction of specificity, variability, self-renewal of protein and origin of life seems very arbitrary and unconvincing. Such a concept is of course extremely simplified.

Combination of amino acids with reactive groups of proteins, demonstrated in the experiments of Pasynsky, Shnol, Konikova and a number of others, is an entirely natural, usual chemical process, in which we see no "contradiction" and "release" of a chemism, "conversion to its opposite", "qualitative leap", "new form of movement of matter", "biological metabolism", "boundary between living and nonliving", "phenomenon of autocatalysis", "self-renewal" and similar properties ascribed to it.

The matter is otherwise with respect to the existence of "self-renewal" of proteins in actual biological systems. At present there are various data indicating the great chemical lability of proteins in the living cell, the presence in protoplasm of not only breakdown and synthesis of whole molecules, but also reactions of replacement in molecules of various amino acid residues and fragments.

In our study of 1952 [1] we saw basic evidence for the existence of self-renewal of proteins without breakdown in the unlike rate of incorporation into proteins of different amino acids, shown in a number of studies [20, 21], which did not correspond to the quantitative ratios of these amino acids in the structure of the proteins. Here we assumed that if some amino acid enters a protein structure only once (during synthesis of the molecule) and once "departs" (during its breakdown), then incorporation of amino acids into proteins should be proportional to their quantitative content in a given protein.

A single explanation of the inequality of "incorporation" of amino acids can not, however, be placed beyond doubt. It is obvious, for instance, that the varying rate of amino acid incorporation into proteins can be the consequence of not only two forms of self-renewal, but also the result of a number of other processes – of unequal dilution of radioactive amino acids in tissues before "incorporation" into proteins due to the varying content in tissues of unlabeled amino acids, of differences in intermediary metabolism of amino acids, of reutilization in protein synthesis of polypeptides already present in the cell, etc.

The most convincing demonstration of the presence in living cells of two forms of protein self-renewal is a series of studies by Gale and Folkes [6] on assimilation of amino acids by bacteria. The object of study was Staphylococcus aureus, which requires for growth a balanced mixture of amino acids.

The authors found that incorporation of a labeled amino acid into proteins can occur as the result of two different processes. The first occurred during incubation of the microbial cells with glucose and C^{14}-glutamic acid. Here cell growth was not observed, but considerable radioactivity was noted in the proteins (~2500 counts/min in protein from 2 mg of dry cell residue). The second process was observed when, during incubation of the bacterial cells besides C^{14}-glutamic acid, the complete amino acid mixture was added. In this case a protein increase was observed and also more active incorporation into proteins of labeled amino acid (up to 8000 counts/min in protein from 2 mg of dry substance). It was found in further studies that the radioactivity of the proteins in both cases was primarily in the structure of the glutamic acid residue (only ~1% entered lysine). A distinct qualitative difference in the two processes was shown on the basis of the different effect on them of a number of factors, in particular: increase of density of the inoculum and effect of various antibiotics (chloramphenicol, aureomycin, bacitracin and penicillin).

In their experiments the authors showed that under the influence of antibiotics in the absence of growth, under the conditions of the first process, various amino acids (13 amino acids were investigated) possess an individual type of incorporation into proteins. The data, in the opinion of the authors, indicate the existence of two paths of incorporation of amino acids into proteins in a living system: in the new synthesis of protein and as a result of exchange reactions, not accompanied by synthesis of new molecules. In subsequent studies [6] Gale and Folkes studied this problem in further detail. Experiments were done on "fragments" of staphylococcus cells obtained by action of ultrasound, deprived of their "internal" solution containing free amino acids. Incorporation of C^{14}-amino acids in the first process was stimulated by ATP, hexose diphosphate and nucleic acid of the staphylococcus. The C^{14}-glutamic acid incorporated into the structure of the protein under these conditions during incubation of cell "fragments" in a medium containing carrier C^{12}-glutamic acid, entered into "exchange" with it, as a result of which the initial relative specific radioactivity of the protein was lowered. This "exchange" was likewise observed only in the presence of a phosphorylating system. No "exchange" occurred when, instead of C^{12}-glutamic acid, C^{12}-glycine or C^{12}-aspartic acid was added to the medium. Calculation showed that such exchange is characteristic of only a small part of the glutamic acid bound in the proteins (5.6%), in connection with which the equilibrium between protein and glutamic acid of the medium was established rapidly – in 30 minutes. In the second process a linear increase of activity in proteins for several hours was observed, accompanied by a corresponding total increase of protein N. Here, however, the rate of incorporation of C^{14}-amino acids in the first 30 minutes was markedly lower than in the first process, indicating the competitive character of the two processes.

Analysis of the data obtained leads the authors to the conclusion that in the first process there occurs an exchange incorporation of amino acids into the preformed proteins by means of substitution, and the significant role of nucleic acids in this process warrants the assumption that such exchange occurs only in nucleoproteins and nucleic acid is a necessary specific factor for such exchange.

In explanation of the unequal dependence of the "exchange" of different amino acids on RNA and DNA the authors propose that "incorporation" of individual amino acids is controlled by specific fragments of nucleic acids, some of these fragments being more easily damaged by ultrasound than others. The utilization of amino acids in self-renewal of proteins was thus determined by interlinkages between portions of proteins and of nucleic acids.

The renewal of the amino acid composition of cell proteins of tumor tissue during inhibition of synthetic reactions was demonstrated recently by Rabinovitz et al. [7]. They studied the relation between phenylalanine, valine, leucine and lysine during their incorporation into proteins of tumor slices. Penetration of the latter three amino acids was studied under conditions where phenylalanine incorporation into proteins was completely inhibited by antagonists. The data obtained indicate that the three indicated amino acids in this case entered the structure of the tumor protein at nearly the original rates. It was typical here that during inhibition of phenylalanine incorporation, accompanied by suppression of synthesis of protein molecules, the incorporation into tumor proteins of valine and leucine was somewhat lowered, while that of lysine scarcely changed. This indicates that the varying rate of exchange of amino acids in proteins, also observed by other authors, is evidently an actual fact, and not the result of various sorts of side phenomena.

All these data, regardless of the correctness of hypotheses proposed for their explanation, show that the biological process of protein self-renewal does not represent simply "self-exchange", but is a complex and specific phenomenon associated with exchange of matter and energy within the cell structures.

Self-renewal of protein without breakdown of macromolecules in living protoplasm is closely interwoven with processes of breakdown, resynthesis and new synthesis of proteins. The mechanism of such self-renewal in many ways is not yet clear, but it must be supposed to be of an enzymic nature.

In the literature, however, facts are described contradicting this conclusion and referring to penetration of amino acids into proteins exclusively during synthesis of the molecules. In work dealing with incorporation of labeled amino acids into proteins of the developing hen's egg, Orekhovich et al. [24] found, for instance, that incorporation of radioactive amino acids into the egg white and yolk proteins occurs only during their synthesis in the body of the hen. During subsequent embryonic development these proteins are no longer renewed. Here, however, the authors were dealing with storage proteins, not taking an active part in intracellular metabolism. Bulman and Campbell [25] did not find "self-renewal" in antibodies taken from immunized rabbits and then introduced into other rabbits. Similar data on antibodies were also obtained by Macheboeuf [26]. There are other analogous studies. But they all, as a rule, are concerned with extracellular or highly specialized proteins (blood albumins and globulins, hemoglobin and certain enzymes, storage proteins, proteins of metaplasm). It therefore seems to us that the conclusion of Orekhovich [27] "that incorporation into proteins of amino acids proceeds only in the process of synthesis of protein substances, and not as a result of their renewal" and "that in the organism there are no renewal processes independent of breakdown and synthesis of the protein molecule" (p. 10), can not be considered proved for all categories of proteins in the organism. For the active intracellular proteins and nucleoprotein complexes under normal conditions this conclusion appears to be unacceptable.

In connection with the data set forth, it seems to us that the controversial problem of the forms of protein self-renewal is being solved by contemporary data. It is obviously necessary to recognize the actual and biologically significant presence of at least two forms of protein self-renewal (complete breakdown and resynthesis and "self-renewal") in metabolically active intracellular proteins, comprising the basic material of the cytoplasmic granules present in direct contact with numerous enzyme systems and nucleic acids and located in the "center" of the exchange of matter and energy. On the other hand, it must also be acknowledged that self-renewal without breakdown is absent or very limited in extracellular and storage proteins, which fulfill other functions and in their basic mass are "cut off" from the various enzyme systems. To this group of proteins belong, for instance, the proteins of milk, egg white and yolk, hair, certain metaplasmic proteins, blood serum proteins, digestive enzymes, protein hormones and others.

With respect to the existence in individual (in the chemical sense) proteins of an inherent property of self-exchange, lying at the basis of "self-renewal" and growth of protoplasm, this must be considered unproved. The criticism of this conclusion, based on the fact that in this case it is difficult to explain the origin of living systems in the first step of development of life, inasmuch as individual proteins incapable of independent exchange can not develop, it is impossible to consider seriously. The authors of this criticism [12, 14, 16] arbitrarily propose that at the sites of synthesis of the first protein there was formed a certain single individual, specific protein, all of whose molecules were identical and each of them capable of growth, multiplication and development of form. Meanwhile, it is entirely obvious that there occurred a formation of diverse proteins with different properties, and their interaction with each other and other compounds in the first coacervates served as the foundation of many processes, as material for the first natural selection. This first natural selection was, as is correctly proposed in the theory of the origin of life developed by A. I. Oparin, the foundation for the further development of life.

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RUSSIAN JOURNALS FREQUENTLY CITED

[Biological Sciences]

Abbreviation*	Journal*	Translation
Agrobiol.	Agrobiologiya	Agrobiology
Akusherstvo i Ginekol.	Akusherstvo i Ginekologiya	Obstetrics and Gynecology
Antibiotiki	Antibiotiki	Antibiotics
Aptechnoe Delo	Aptechnoe Delo	Pharmaceutical Transactions
Arkh. Anat. Gistol. i Embriol.	Arkhiv Anatomy, Gistology i Embriology	Records of Anatomy, Histology and Embryology
Arkh. Biol. Nauk SSSR	Arkhiv Biologicheskikh Nauk SSSR	Records of Biological Science USSR
Arkh. Patol.	Arkhiv Patology	Records of Pathology
Biofizika	Biofizika	Biophysics
Biokhimiya	Biokhimiya	Biochemistry
Biokhim. Plodov i Ovoshchei	Biokhimiya Plodov i Ovoshchei	Biochemistry of Fruits and Vegetables
Bot. Zhur.	Botanichesky Zhurnal	Journal of Botany
Byull. Eksptl. Biol. i Med.	Byulleten Eksperimentalnoi Biology i Meditsiny	Bulletin of Experimental Biology and Medicine
Byull. Moskov. Obshchestva Ispytatelei Prirody, Otdel Biol.	Byulleten Moskovskogo Obshchestva Ispytatelei Prirody, Otdel Biologichesky	Bulletin of the Moscow Naturalists Society, Division of Biology
Doklady Akad. Nauk SSSR	Doklady Akademy Nauk SSSR	Proceedings of the Academy of Sciences USSR
Eksptl. Khirurg.	Eksperimentalnaya Khirurgiya	Experimental Surgery
Farmakol. i Toksikol.	Farmakologiya i Toksikologiya	Pharmacology and Toxicology
Farmatsiya	Farmatsiya	Pharmacy
Fiziol. Rasteny	Fiziologiya Rasteny	Plant Physiology
Fiziol. Zhur. SSSR	Fiziologichesky Zhurnal SSSR im. I. M. Sechenova	I. M. Sechenova Physiology Journal USSR
Gigiena i Sanit.	Gigiena i Sanitariya	Hygiene and Sanitation
Izvest. Akad. Nauk SSSR, Ser. Biol.	Izvestiya Akademy Nauk SSSR, Seriya Biologicheskaya	Bulletin of the Academy of Sciences USSR, Biology Series
Izvest. Tikhookeanskogo N. I. Inst. Rybnogo Khoz. i Okeanog.	Investiya Tikhookeanskogo N. I. Instituta Rybnogo Khozyaistva i Okeanografy	Bulletin of the Pacific Ocean Scientific Institute of Fisheries and Oceanography
Khirurgiya	Khirurgiya	Surgery
Klin. Med.	Klinicheskaya Meditsina	Clinical Medicine
Lab. Delo	Laboratornoe Delo (po Voprosam Meditsiny)	Laboratory Work (on Medical Problems)
Med. Parazitol.	Meditsinskaya Parazitologiya i Parazitarnye Bolezni	Medical Parasitology and Parasitic Diseases
Med. Radiol.	Meditsinskaya Radiologiya	Medical Radiology
Med. Zhur. Ukrain.	Medichny Zhurnal Ukrainsky	Ukrainian Medical Journal
Mikrobiologiya	Mikrobiologiya	Microbiology
Mikrobiol. Zhur.	Mikrobiologichny Zhurnal	Microbiology Journal
Nevropatol., Psikhiat. i Psikhogig.	Nevropatologiya, Psikhiatriya i Psikhogigiena	Neuropathology, Psychiatry and Psychohygiene
Ortoped., Travmatol. i Protez.	Ortopediya, Travmatologiya i Protezirovanie	Orthopedics, Traumatology and Prosthetics
Parazitol. Sbornik	Parazitologichesky Sbornik	Parasitology Collection
Pediatriya	Pediatriya	Pediatrics
Pochvovedenie	Pochvovedenie	Soil Science
Priroda	Priroda	Nature
Problemy Endokrinol. i Gormonoterap.	Problemy Endokrinology i Gormonoterapy	Problems of Endocrinology and Hormonal Therapy
Problemy Hematol.	Problemy Hematology i Perelivaniya Krovi	Problems of Hematology and Blood Transfusion
Problemy Tuberk.	Problemy Tuberkuleza	Problems of Tuberculosis
Sovet. Med.	Sovetskaya Meditsina	Soviet Medicine
Sovet. Vrachebny Zhur.	Sovetsky Vrachebny Zhurnal	Soviet Physicians Journal
Stomatologiya	Stomatologiya	Stomatology
Terap. Arkh.	Terapevticheskiy Arkhiv	Therapeutic Records
Trudy Gelmint. Lab.	Trudy Gelmintologicheskoi Laboratoriya	Transactions of the Helminthology Laboratory
Trudy Inst. Genet.	Trudy Instituta Genetiki	Transactions of the Institute of Genetics
Trudy Inst. Genet. i Selektsy	Trudy Instituta Genetiki i Selektsy	Transactions of the Institute of Genetics and Plant Breeding

* Consultants Bureau Transliteration System.

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Abbreviation	Journal	Translation
Trudy Inst. Gidrobiol.	Trudy Instituta Gidrobiologiya	Transactions of the Institute of Hydrobiology
Trudy Inst. Mikrobiol.	Trudy Instituta Mikrobiologiya	Transactions of the Institute of Microbiology
Trudy Inst. Okean.	Trudy Instituta Okeanologiya, Akademy Nauk SSSR	Transactions of the Institute of Oceanology, Academy of Sciences, USSR
Trudy Leningrad Obshchestvo Estestvo.	Trudy Leningrad Obshchestvo Estestvoispytately	Transactions of the Leningrad Society of Naturalists
Trudy Vsesoyuz. Gidrobiol. Obshchestva	Trudy Vsesoyuznogo Gidrobiologicheskogo Obshchestva	Transactions of the All-Union Hydrobiological Society
Trudy Vsesoyuz. Inst. Eksptl. Med.	Trudy Vsesoyuznogo Instituta Eksperimentalnoi Medistiny	Transactions of the All-Union Institute of Experimental Medicine
Ukrain. Biokhim. Zhur.	Ukrainsky Biokhimichny Zhurnal	Ukrainian Biochemical Journal
Urologiya	Urologiya	Urology
Uspekhi Biokhimiya	Uspekhi Biokhimiya	Progress in Biochemistry
Uspekhi Sovremennoi Biol.	Uspekhi Sovremennoi Biologiya	Progress in Contemporary Biology
Vestnik Akad. Med. Nauk SSSR	Vestnik Akademy Meditsinskikh Nauk SSSR	Bulletin of the Academy of Medical Science USSR
Vestnik Khirurg. im. Grekova	Vestnik Khirurgy imeni Grekova	Grekov Bulletin of Surgery
Vestnik Leningrad. Univ. Ser. Biol.	Vestnik Leningradskogo Universiteta, Seriya Biology	Journal of the Leningrad Univ., Biology Series
Vestnik Moskov. Univ., Ser. Biol. i Pochvov.	Vestnik Moskovskogo Universiteta, Seriya Biology i Pochvovedeniya	Bulletin of the Moscow University, Biology and Soil Science Series
Vestnik Oftalmol.	Vestnik Oftalmology	Bulletin of Ophthalmology
Vestnik Oto-rino-laringol.	Vestnik Oto-rino-laringologiya	Bulletin of Otorhinolaryngology
Vestnik Rentgenol. i Radiol.	Vestnik Rentgenology i Radiology	x-Ray and Radiology Bulletin
Vestnik Venerol. i Dermatol.	Vestnik Venerology i Dermatology	Bulletin of Venerology and Dermatology
Veterinariya	Veterinariya	Veterinary Science
Vinodelie i Vinogradarstvo	Vinodelie i Vinogradarstvo	Viticulture and Wine-making
Voprosy Klin.	Voprosy Klinichesky	Clinical Problems
Voprosy Med. Khim.	Voprosy Meditsinskoi Khimy	Problems of Medical Chemistry
Voprosy Med. Virusol.	Voprosy Meditsinskoi Virusology	Medical Problems of Virusology
Voprosy Neirokhirurg.	Voprosy Neirokhirurgy	Problems of Neurosurgery
Voprosy Onkol.	Voprosy Onkology	Problems of Oncology
Voprosy Pitaniya	Voprosy Pitaniya	Problems of Nutrition
Voprosy Psikhology	Voprosy Psikhology	Problems of Psychology
Voprosy Virusology	Voprosy Virusology	Problems of Virusology
Vrachebnoe Delo	Vrachebnoe Delo	Medical Profession
Zav. Lab.	Zavodskaya Laboratoriya	Factory Laboratory
Zhur. Mikrobiol., Epidemiol. i Immunobiol.	Zhurnal Mikrobiology, Epidemiology i Immunobiology	Journal of Microbiology Epidemiology and Immunobiology
Zhur. Nevropatol. i Psikhiat.	Zhurnal Nevropatology i Psikhiatry imeni S. S. Korsakov	S. S. Korsakov Journal of Neuropathology and Psychiatry
Zhur. Obshchei Biol.	Zhurnal Obshchei Biologiya	Journal of General Biology
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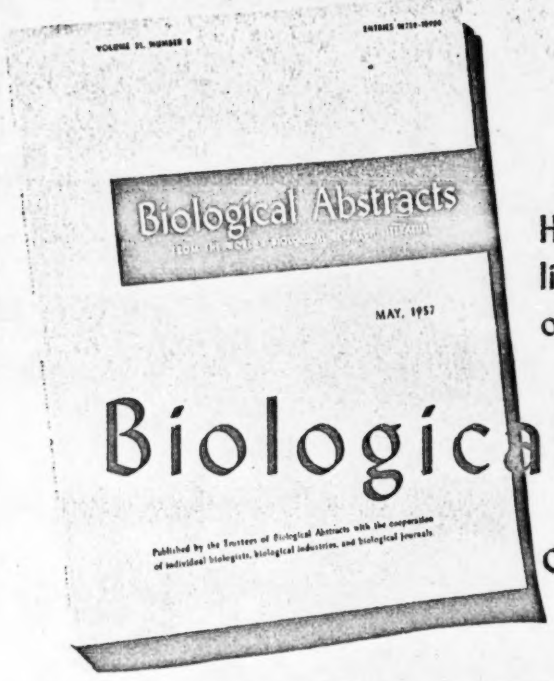
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